ABSTRACT

BIOLOGY

HARRIS, SANDRA A.

B.S., Dillard University, 1974

M.S., Atlanta University, 1976

Cadmium Toxicity in the Kidneys and Liver of 4-7 Day Chicken Embryos: Histological, Histochemical and Atomic Spectrophotometric Analysis

Advisor: Dr. Roy Hunter, Jr.

Doctor of Philosophy degree conferred August 4, 1978

Thesis dated August, 1978

Histological and histochemical studies on embryogenesis in normal, cadmium-treated and selenium-treated chick embryos from days 4-7 were observed. An atomic absorption spectrophotometric study of cadmium concentrations in liver and kidneys of normal and cadmium-treated embryos has also been made. These studies revealed that cadmium causes damages in tubules and glomeruli of the chick mesonephros. Selenium was also found to disarrange tubular epithelium but destruction in the majority of selenium-treated embryos was not as great as that of cadmium.

Acid phosphatase localization in the cadmium-treated five-day embryos was much reduced by comparison with the seven-day-old specimens. Alkaline phosphatase activity was much more intense in the brush borders of proximal tubules of controls than in any stage of experimental embryos. Beta-glucuronidase activity was slightly positive in the

tubular epithelium, becoming progressively stronger toward the lumen of the tubule. As in the case of alkaline phosphatase, the cytochrome oxidase activity was more pronounced in the control sections than in the experimental.

The concentration of cadmium in the mesonephros of cadmium-treated embryos was three to four times as much as that in the liver. Data presented indicate that cadmium disrupts developmental enzyme activities and that cadmium concentrates predominantly in the kidneys.

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CADMIUM TOXICITY IN THE KIDNEYS AND LIVER OF 4-7 DAY CHICKEN EMBRYOS: HISTOLOGICAL, HISTOCHEMICAL AND ATOMIC SPECTROPHOTOMETRIC ANALYSIS

A THESIS

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SANDRA A. HARRIS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

The rapidly increasing store of information on the distribution of heavy metal levels in the environment has not brought about a concomitant understanding of the means by which these metals are stored in biological systems. Cadmium, a heavy metal and environmental pollutant, was discovered in 1817 by the German chemist, F. Strohmeyer (1778-1835). During a mineralogical examination of some zinc ores, his attention was drawn to the yellow color of one of the samples of smithsonite, which in the pure form is white.

The results to be described here grew out of an investigation into the effects of small doses of cadmium chloride on the kidneys and liver of chicken embryos (Harris, 1976). The kidneys and liver from cadmium-treated chicken embryos were macroscopically and microscopically examined. The morphological changes seen in these organs, it was thought, warranted a more extended investigation.

Therefore, the objectives of this research were to make: (1) a detailed histological study of the developing kidney (mesonephros); (2) a histochemical examination of acid and alkaline phosphatase, beta-glucuronidase, and cytochrome oxidase activity, and (3) an analysis of cadmium concentration in the kidneys and liver of chick embryos by flame absorption spectrophotometry.

CHAPTER II

REVIEW OF LITERATURE

Metals have been known for some time to play an important role in a wide variety of biological processes. Many specific plant, animal and human disease syndromes are due to the relative excess or deficiency of a single metal (Motto et al., 1970; Mottet, 1974). In man, some of these syndromes may be the result of genetically determined abnormalities in metabolic events in which metals play an essential part, while others follow environmental exposure to metals from manufacturing or other industrial uses with subsequent toxic results. In addition some metals have long been known to be oncogenic. They may induce a wide variety of tumors, either as a result of experimental study in a variety of laboratory animals, or as a result of accidental exposure and toxic accumulation in man (Ferm, 1974).

For all practical purposes, the presence of metals in mammalian tissues can be attributed to the fact that either they are required for some essential metabolic function or that they represent an accumulation from accidental exposure, whatever the source. Schroeder (1960) has put it simply: any element not present in plants is not essential for man and any element found in man but not in plants, is an environmental contaminant.

Many parallelisms exist between teratogenesis and carcinogenesis: (1) Somatic cell division is a prerequisite for both. (2) Of the number of compounds which have been bioassayed for both effects, the majority have produced malformations and cancer. (3) The same compound may have

different effects, dependent upon the stage of development of the organism at the time of exposure. Thus, a compound found to be carcinogenic to mature cells might be teratogenic to immature embryonic cells. Certain metals have had clear-cut teratogenic effects on mammalian embryonic development. There is no apparent relationship among these elements to explain their teratogenic effect, but all of them do represent potentially serious environmental pollutants, one of these being cadmium.

Cadmium (Cd) compounds have been known for over a century to be toxic to man, and the symptomology accompanying both acute and chronic intoxication has been described (Johnson et al., 1975). In earlier years Cd was largely recognized as an industrial hazard, but recent attention has been directed toward the role of trace levels of Cd as an etiological factor in various chronic pathological conditions such as arteriosclerosis, hypertension, testicular tumors, renal dysfunction, emphysema, growth inhibition and cancer. Cadmium is widely spread throughout the biosphere and has currently become recognized as a toxicologically important environmental contaminant. In the United States, the daily human intake of this metal has been reported to be 200,500 ug (Johnson et al., 1975).

At present, atomic absorption is the most commonly used method for the determination of cadmium. An atomic absorption spectrophotometric method for the direct determination of zinc in biological fluids has been developed, which does not require destruction or removal of organic material (Fuwa et al., 1964). The limit of detection is 0.002 ug of Zn per ml.

Evenson and Anderson (1975) describes a method of analysis for Cu,

Cd, and Zn in a 15-mg (wet weight) sample of human liver by atomic spectrophotometry. For 16 histologically normal samples of human liver, the mean values were Cu, 26; Zn, 293: and Cd, 6.0 nanograms of metal per milligram dry weight.

Another technique for atomic absorption determinations of Zn, Cu, and Cd, in tissues has been devised. The tissue is solubilized by aqueous tetramethammonium hydroxide. Murthy and his associates (1973) proposed that this method allows faster and safer processing and handling of samples in comparison to acid digestion procedures.

Cadmium is closely related to zinc (Zn) and will be found wherever Zn is found in nature. The Cd to Zn ratios range from 1:100 to 1:1000. Zinc is an essential metal for most life forms (Underwood, 1962; Bowen, 1966; Schroeder et al., 1967; Yamagata and Shigematsu, 1970). Thus, it is probable that no naturally occurring material will be completely free of cadmium. This metal is obtained as a by-product in the refining of Zn and other metals. However, since it is difficult to separate Zn and Cd, the latter will often be found in small amounts in commercially available Zn compounds (Schroeder et al., 1967).

Though cadmium has been recognized for only a comparatively short period of time, copper, lead, zinc and some other metals have been used for several thousand years (Catizone and Gray, 1941; Heath, 1949). As soon as man started to produce metals, he also started to pollute the environment with cadmium. In this century Cd compounds have been used increasingly by industries, causing a sharp increase in environmental contamination. Cadmium will be emitted to air and water by mines, by

metal smelteries, and plastics. The burning of oil and waste and scrap metal treatment will also contribute. The use in agriculture of fertilizers, either as chemicals or as sludge fromsewage plants, and the use of Cd-containing pesticides might also contribute to the contamination.

Some of the Cd emitted to air will be inhaled by people and animals but most of it will be deposited in soil or water. The Cd deposited in water may then increase the concentration of Cd in edible water organisms. In the event of flooding or irrigation, Cd in water might also increase the concentration in soil, inturn causing an increase in agricultural products, such as rice and wheat (Friberg et al., 1974). Therefore, the five basic routes of exposure are: (1) air, (2) water, (3) soil and uptake by plants, (4) food and (5) cigarettes.

Over 90 cases have been reported on poisoning which resulted from a high concentration of Cd fumes liberated during heating in welding or flanging of Cd-plated metal. The symtoms are constriction in the throat, pains in the chest and severe dyspnea due to pulmonary edema and congestion. The first reports of chronic Cd poisoning (Friberg, 1950) dealt with men exposed to Cd oxide dust when working in alkaline accumulators. The clinical findings were anoxmia, dyspnea due to emphysema and a proteinuria in which the protein has a characteristic low molecular weight. Similar symtoms were reported (McQueen, 1951; Lane and Campbell, 1954) in cases where men were repeatedly exposed to brief but high concentrations of Cd fumes in the manufacture of a Cd-Cu alloy.

The initial site of deposition of cadmium was the lungs into which the fumes were inhaled. From this site the Cd becomes widely distributed, with the metal accumulating predominantly in the liver and kidneys. Too, significant amounts were demonstrated in the bile and urine, indicative of slow excretion. The greatest problem in these cases was the pathogenesis of the emphysema. This condition is common in the industrial areas in which these men were employed, and it was very difficult to establish that their emphysema was in any way different from that in the general population. Clinically, most cases of emphysema gave a history of cough and sputum, suggestive of chronic bronchitis; others suffered from respiratory destruction due to asthma, and some had a persistent cough from bronchiestasis. Thurlbeck and Foley (1971) injected a 1% aqueous solution of $CdCl_2$ into the trachea of guinea pigs. This induced an acute, hemorrhagic, edematous reaction, often with hyaline membranes. Most of the animals died during this acute phase. The surviving animals showed marked fibroblastic changes in the parenchyma that evolved to severe peribronchial fibrosis, with associated alveolar destruction and distortion, which Thurlbeck and Foley likened to scar emphysema. Snider et al., (1973) noted acute vascular congestion and alveolar hemorrhage, followed by polymorphonuclear cell infiltration, after exposing rats to an aerosol of $CdCl_2$ in saline for varying periods of time. The mechanism of Cd clearance from the lung is unclear, but various studies (Harrison et al., 1947) suggest that Cd was absorbed in the lung and transported to the liver, an organ known to be rich in the metal binding protein fraction, metallothionein (Piscator, 1964).

In recent years, the chronic effects of Cd on health have attracted the special attention of both the medical profession and the general public in Japan. This attention has been aroused because several investigators have reported that edemic "Itai-itai" disease, meaning "ouch-ouch" disease, which occurred among the inhabitants of a localized area in Toyama prefecture, is a kind of chronic Cd poisoning (Hagino, 1961; Ishizaki et al., 1965). Such attention has been further stimulated by the fact that the soil, river water, crops, and marine products in other areas are also contaminated with the metal if Zn or Cd refineries are located in them or in their vicinity (Yamamoto, 1972).

Cadmium that is consumed from foods and drinking water, along with coexisting nutritional deficiency, have been shown to play a primary role in the pathogenesis of"Itai-itai" disease. The disease is essentially a renal lesion similar to Faconi syndrome (Axelsson and Piscator, 1966; Axelsson et al., 1968; Piscator and Axelsson, 1970; Nomiyama et al., 1973; Nomiyama et al., 1975). However, the disease is also accompanied by severe damage to bones (Ishizaki et al., 1966; Kobayashi, 1971; Itokawa et al., 1973; Itokawa et al., 1974; Yoshiki et al., 1975).

The clinical course of the disease has been described by several investigators (Nakagawa, 1960; Takase et al., 1967; Hagino, 1968a, 1968b, 1969; Ishizaki, 1969b; Tsuchuja, 1969; Murata et al., 1969, 1970). The most characteristic features of the disease are lumbar pains and leg myalgia. Pressure on bones, especially the femurs, backbone, and ribs,

produced further pain. Another characteristic of the disease is a duck-like gait. Such conditions continue for several years until one day the patient experiences a mild trauma and is unable to walk.

The morphology of bone marrow in Cd intoxicated rabbits prompted an investigation of earlier reports which showed Cd poisoning as not inhibiting hemoglobin synthesis in rabbits (Berlin, Fredricsson and Linge, 1961).

To the contrary, some acceleration of synthesis was evident. Hypochronic anemia with microcytosis occurred in all rabbits injected with cadmium. Histologically, the ordinary pattern of bone marrow was altered by Cd; normal and even distribution of myelopoietic and reticular cells between the fat cells was lost, and the stroma of the bone marrow was grossly changed.

A statistical analysis has been performed on the results of the epidemio-study for Itai-itai disease that was carried out in 1967 by Toyama Precture Health Authorities (Kato and Kawano, 1968).

The investigators found close dose response relationships between Cd exposure and health effects, including a variation with age in their estimation of daily Cd intake based on an analysis of Cd in feces and urine.

Several clinical observations have been reported on renal lesions caused by prolonged exposure to Cd dust in industries (Friberg, 1950, 1959), and high Cd contents have actually been demonstrated for the affected organs, such as the liver and kidney (Friberg, 1950, 1957). In

two autopsies, Kajikawa et al., (1957) found senile arteriosclerotic contracted kidneys, pyelonephritis, and metastatic calcification. In the tubules, atrophic changes with flattening of the epithelium were seen, Harris (1976) also noted morphological destruction of the kidneys in chicken embryos exposed to cadmium chloride. A frequent finding after Cd poisoning was degeneration of the periportal region of the liver lobules. Damage to the kidney was most apparent in the tubules, which showed pronounced eosinophilia and nuclear degeneration. Similar observations were reported in rats (Pindborg and Plum, 1946) and mice (Berlin and Ullberg, 1963). A study of the toxic effects of Cd administration on the kidney and bone in four groups of rats has been conducted recently by (Itowaka et al., 1978). Two groups were maintained on a diet sufficient in calcium and two on a diet deficient in calcium. One group receiving each diet was administered $CdCl_2$ (0.02%) in the diet. These rats showed an increase in blood urea nitrogen and serum phosphorus levels; a decrease in the clearance of inulin, phosphorus, and calcium, and an increase in the fractional excretion of calcium with an absence of a significant change in the tubular reabsorption of phosphorus.

Cousins, Barber and Trout (1973) reported in their study on cadmium toxicity in swine that the ability of the kidney to handle protein was diminished. Renal protein catabolism was undoubtedly impaired as the activity of kidney leucine aminopeptidase (LAP) was inhibited in those pigs receiving 150 ppm or more dietary cadmium; serum leucine aminopeptidase was not affected. Recently Mogielnicki et al., (1973) proposed that small proteins are normally, in most cases,

filtered through the glomeruli and taken up and catabolized in the tubular cells. In tubular proteinuria, tubular protein uptake and catabolism decreased, thus allowing more protein to appear in the urine. Using this analogy, if Cd bound as Cd-binding-protein (CdBP) is normally filtered and then catabolized, freed Cd would exchange with the Zn metalloenzyme, LAP, alter renal proteolytic activity, and preclude further CdBP increases in the kidney.

Parizek (1962) was the first to report that Cd salts, when injected subcutaneously into the rat, produced a rapid and selective destruction of the testis. Kar and Das (1962) found that the administration of $CdCl_2$ directly into the rat testis caused acute and irreversible destruction of the germinal epithelium. The interstitial portion regenerated after an initial phase of atrophy. These effects were restricted to the treated testis only; the contralateral one remained unaffected.

This damage was so extensive in the testicular cells of rats that Cameron and Foster (1963) were prompted to investigate along similar lines in the rabbit. In reactive animals (rabbits) Cd produced marked swelling and discoloration of the testis. The color, which was due to extravasation of blood into the interstitium ranged from a light reddish hue appearing on the first day after initial injection, to purple or a dark mahogany shade on the second and third days.

Chiquoine (1963) noted that testicular necrosis following Cd administration does not occur, however, in all vertebrates. Employing dosages as high as 1 mg $CdCl_2$/kg, no testicular necrosis was observed

after intervals of one, two, and three days in the grass frog, pigeon, rooster, armadillo, or opossum. A light and electron microscopic study of the early changes which occurred in Cd necrosis of the testis in mice was made in an effort to identify the site of action of Cd (Chiquoine, 1964; Nishizumi, 1972). The earliest changes detected by light microscopy consisted of an edema of the interstitial spaces, congestion of blood vessels and an increased amount of granular precipitate in the connective spaces. The fact that the earliest alterations were observed in blood vessels led this investigator to suggest that the site of Cd in the production of testicular necrosis was the endothelium of the vascular bed.

The early changes in the microvascular bed of the testis resulting from treatment with Cd have also been studied using trypan blue, electron dense tracers and vascular injections (Aoki, 1978). Leakage of fluids and electrolytes from the testicular blood vessels into the interstitium was demonstrated as early as 1-2 hr following parenter injection of Cd. By 3 hr the extravasation of carbon had increased, such that particles could be seen labeling veins. At 4 hr a striking decrease in labeling of veins was noted due to the fact that delivery of the tracer toward the venous system is almost entirely obstructed as a result of the blockage and progressive deterioration of the smaller vessels; ischemia of the testis ensued from the obstruction of the microvascular circulation. The indication is that the degeneration of the seminiferous epithelium and all biochemical and physiological changes known to occur in the testis at later time intervals following Cd treatment, are secondary to ischemia rather than due to a direct effect of cadmium.

Studies on the placental transfer of metals are meager, especially during the critical phase of development. Using radioautographic techniques, Berlin and Ullberg (1963) postulated that there was no placental transfer of Cd in the pregnant mouse although selective areas of the placenta appeared to concentrate it. Contrary to this hypothesis, studies utilizing this same isotope in pregnant golden hamsters revealed that radioactive Cd crosses the placenta with ease during the early critical stages of organogenesis (Ferm et al., 1969). Their studies, however, were supported by Parizek (1964), who compared the effects of low doses of Cd in three groups of rats: pregnant females, nongravid females and females given Cd salts within 3 days after giving birth. In contrast to the good survival of nongravid rats, the injection of Cd salts into gravid rats was followed by a high mortality (76%) within 1 to 4 days after injection. The first sign of illness in some of the injected gravid rats was the appearance of blood in the urine within 6 hr post-injection.

Autopsy of rats that died within 24 hr of injection revealed visceral venous congestion, hemorrhagic edema and swollen kidneys, with hemorrhaging in the renal medulla. Upon administering Cd sulfate into pregnant hamsters on day 8 of gestation, Gamm and Ferm (1970) noted marked deleterious effects on the mesoderm of the embryos. As a result numerous malformations occurred, including unilateral and bilateral cleft lips and plates.

Lauwerys et al., (1978) investigated the placental transfer of lead, mercury, cadmium, and carbon monoxide in women. Comparison of the frequency distributions of the various hematological indices in maternal

and umbilical cord blood indicated that the three heavy metals are transferred from the mother to the fetus; however, the barrier role of the placenta is different for the three metals. There is no barrier for the transfer of mercury, a slight one for lead, and a more important one for cadmium. This explains why the correlation found between Cd concentrations in maternal and fetal blood is much lower than that found for lead and mercury.

A trace metal which accumulates in a metabolic organ with age may be suspected of influencing the development of an age-linked disorder. Schroeder and Balassa (1961) and Scroeder and Vinton (1962) observed that female rats on a Cd-free diet exhibited fluctuating systolic hypertension when given Cd in drinking water at subtoxic levels from the time of weaning up to 180-240 days. Relatively small accumulations of Cd in the kidney and liver were present, and hypertension was the only sign of toxicity. Because this method was time consuming and involved extensive precautions to avoid contamination by Cd other than in drinking water, an attempt was made to induce hypertension in rats by the injection of Cd and to compare the severity of Cd-induced hypertension by a more conventional method.

Cadmium acetate was injected into rats at dosages of 2 mg/kg. Arterial hypertension resulted, at incidences similar to or exceeding those resulting from partial constriction of one renal artery (Schroeder etal., 1966). Cadmium is the only trace metal which can produce in the rat the clinical and pathological picture of hypertensive disease. The in vivo mechanism of acute hypertensive effects of Cd has been studied

by Thind et al., (1970, 1973). They (1973) found in an in vitro study of the direct effects of Cd ions on spirally-cut strips of rabbit thoracic aorta, that Cd was a mild direct vasopressor and potent inhibitor of angiotensin, epinephrine, and norepinephrine responses.

Although Cd has not been shown to be essential for plant growth and development, its potential accumulation and effect upon plant nutrition is a concern of plant scientists, as is its effect upon animals that consume the plants. Linnman (1973) demonstrated that as the concentration of Cd increased in soybeans (Glycine max L.) and wheat (Triticum aestivum), dry matter yield decreased. Other workers reported that over a short period of time (3 to 12 days) the Zn concentration of corn shoots and roots decreased with an increase in Cd rate, and the Fe content increased (Root et al., 1975).

As a result of Hart and Scaife's (1977) efforts to determine the toxicity and bioaccumulation of Cd in Chlorella, a number of interesting facts concerning fresh-water algae has been revealed. Chlorella pyrenoidosa cultures, grown at pH 7 in the presence of 1.00 mg of Cd/l had doubling times of 11, 21, 22, 35 h, respectively. Similar exposed cultures grown at pH 8 had doubling times of 11, 16, 17 and 25 hr respectively. The ability of phytoplankton to accumulate and retain large concentrations of Cd before showing adverse physiological effects could have important environmental implications, since phytoplankton form the base of the food chain and are directly consumed by filter-feeding invertebrates.

From a physiological point of view, Meek (1959) reported that Cd

is clearly related to Zn; that its administration might, therefore, interfere with the action of Zn to some extent. In a study by McBean et al., (1972), the Zn concentration in tissue of 30 adult subjects at necropsy from two broad categories of disease and nondisease (sudden accidental death) were determined. Zinc concentration of the bone, muscle, liver, pancreas, and kidney did not reveal any statistically significant differences between these two groups.

The chemical similarities between copper, zinc, and cadmium led to the speculation that there existed a copper component of Cd toxicity in addition to the previously shown zinc component. Cadmium was found to be toxic to chicks at dietary levels of 25 to 400 ppm in a copper- and iron-deficient diet. Growth depression and gizzard abnormality were corrected by increased dietary Zn. The mortality was reversed by added copper, while iron partially corrected both the mortality and the growth depression, indicating a suspected iron component of Cd toxicity.

Selenium compounds are effective in preventing destruction of the testis by subcutaneous administration of cadmium. Zinc, cobalt, and cysteine also block the lethal effects of large doses of cadmium. Considering the ratio of protective agent to Cd which is needed, selenium and Zn are relatively more efficient in protecting against lethality than preventing testicular injury from Cd (Kar et al., 1960).

Reports from several sources have suggested that selenium may also affect carcinogenesis (Shamberger et al., 1970; Harr et al., 1973). Epidemological studies relate an increased incidence of colon cancer among humans in geographical regions where selenium is deficient and a

lowered cancer incidence at enhanced selenium levels (Shamberger et al., 1971).

Cadmium's mode of action is of practical interest as well as of theoretical importance. Investigations by Simon et al., (1947) indicated that Cd reacts with tissue proteins, causing alteration in structure or decrease in solubility sufficient to impair enzyme activity, thereby making cellular function impossible. Combinations between Cd and -SH groups were readily demonstrable but neither this factor nor the formation of insoluble Cd-proteinate can completely explain Cd toxicity to various organs. Some -SH containing enzymes are not affected by Cd in concentrations that completely inhibit others, nor do some other heavy metals share the drastic effects of Cd (Tobias et al., 1946).

Glutathione peroxidase (GSH-PX) activity was elevated in damaged testes of rats whose Cd uptake exceeded a level of approximately 150 ng/g. In rats whose testes were not damaged, the Cd levels were below 150 ng/g and the GSH-PX activity was similar to that of control animals injected with sodium acetate. The direct relationship of hydrolytic enzymes to cell death in most embryonic systems still remains a perplexing problem. In studies by Wilson and Allenspach (1974) and Allenspach (1976), the roles of some hydrolytic enzymes during esophageal organogenesis in the developing chick was examined. Results showed a biochemical increase in hydrolytic enzymes which correlated closely with the period of extensive cellular death.

CHAPTER III

MATERIALS AND METHODS

General Procedure

Fertile eggs of White Leghorn chickens were incubated in a humid atmosphere at 37° C. Prior to incubation, the eggs were swabbed with 70% ethyl alcohol. Controls and experimental series were established to demonstrate the effects of cadmium on chicken embryo development.

After 48 hr of incubation, 0.25 cc of 0.025 M cadmium chloride (Fisher Brand) was injected into the air sac of each eggs; the opening sealed with tape, and all eggs returned to the incubator. Selenium (0.25 cc of 0.025 M sodium selenite) was injected in the same manner as cadmium into 48 hr embryos. All eggs used as controls were injected with 0.25 cc of a 0.85% saline solution and observed grossly. A total of 303 control and 927 experimental eggs was used. The eggs were re-opened at various time intervals (4th, 5th, 6th and 7th day of incubation).

Histological Procedure

For histological examination, the embryos were fixed in 10% buffered formalin. In preparation for paraffin embedding, the embryos were submerged in a standard series of increasing strength alcohol solutions and then in xylene. The infiltration procedure consisted of replacing xylene with a warm paraffin/xylene solution, soft paraffin

and hard paraffin. The embryos were then placed in plastic embedding molds already filled with melted hard paraffin. The embedding molds were placed in cold water to facilitate rapid cooling. The tissue was sectioned at 10 u and stained with Delafield's hematoxylin and eosin-orange G (Humason, 1967). Histological observations were made and photographs of representative sections were taken using an American Optical photo-micrographic set-up.

Histochemical Procedures

Fresh tissue was cut in a Tissue-Tex II Microtome/Cryostat at 15°C. Frozen sections of 7 u were utilized throughout the experiment. Histochemical observations were made and photographs of representative sections were taken as previously described.

Acid Phosphatase

For demonstration of acid phosphatase a modification of the Napthol As-Mx phosphate method of Kaplow and Burstone (1964) was used. (Sigma Technical Bulletin No. 385). The procedure for staining and counter-staining was as follows:

1. Add 2 ml of Napthol As-Mx phosphate Acid solution to 48 ml of distilled water (37°C).
2. Add the contents of a Fast Blue RR Salt capsule and mix throughly. Filter rapidly through coarse paper into a Coplin jar to remove undissolved dye.
3. Immediately immerse slides in dye-substrate solution and incubate for 24 hr.
4. Remove slides and wash gently with tap water.

5. Counter-stain by immersing slides in Mayer's Hematoxylin solution for 2 min.

6. Wash slides with tap water for 20 sec and allow to air dry.

7. Mount slides in Glycerol-Gelatin.

8. Positive results are indicated by brown-black deposits.

9. Control slides are processed by the same procedure as test slides but without Naphthol Acid Phosphate in the incubation medium.

Alkaline Phosphatase

The technique used for demonstration of alkaline phosphatase is a modification of that described by Ackerman (1962). (Sigma Technical Bulletin, No. 85). The procedure for staining and counter-staining was as follows:

1. Add 2 ml of Napthol Ms-Mx Alkaline solution to 48 ml of distilled water.

2. Add the contents of a Fast Blue RR Salt capsule and mix throughly for 30 sec.

3. Filter rapidly through coarse paper into a Coplin jar to remove undissolved dye.

4. The slides are immediately immersed into alkaline-dye mixture and incubated at room temperature for 60 min.

5. Remove slides and wash gently with tap water.

6. Counter-stain by immersing slides in Mayer's Hematoxylin solution.

7. Control slides are processed by the same procedure as test slides, but without Naphthol Alkaline Phosphatase in the incubation medium.

Beta-Glucuronidase

For demonstration of beta-glucuronidase a modification of Gurr's technique (1960) was used. The procedure for staining and counter-staining was as follows:

1. Immerse frozen sections in cold 0.1 M acetate buffer, pH 5.2.
2. Incubate in reagent E (1 ml of acetate buffer; 13 ml of 8-hydroxyquinoline glucuronide in acetate buffer; 13 ml of 8-hydroxy quinoline in acetate buffer, and 9 ml of ferric sulfate) for 24 hr at 37°C.
3. Wash briefly in distilled water.
4. Immerse slides in reagent G (Oxalate buffer) for 15 min.
5. Wash in distilled water.
6. Immerse slides in reagent H (Potassium ferrocyanide) for 15 min.
7. Wash indistilled water.
8. Counter-stain in reagent I (Neutral Red-Carbol Fuschin) for 1 min.
9. Wash in distilled water.
10. Dehydrate through the usual graded alcohols.
11. Clear in xylene and mount.
12. Sites of beta-glucuronidase are indicated by a precipitate of Prussian blue.
13. Control slides are processed by the same procedure as test slides, but incubation is done in reagent F (Reagent E containing potassium hydrogen saccharate).

Cytochrome Oxidase

For demonstration of cytochrome oxidase a modification of the method of Burstone (1962) was used. (Sigma Technical Bulletin No. 185). The procedure for staining and counter-staining was as follows:

1. Add 0.5 ml of ethanol to a 50 mg ampule of 8-Amino-1,2,3,4-Tetrahydroquinoline to dissolve contents. Transfer solution to Erlenmeyer flask.
2. Add 15 mg of p-Aminodiphenylamine and swirl to dissolve.
3. Add 35 ml distilled water and 15 ml Trizma buffer. Centrifuge and transfer supernatant fluid into a Coplin jar.
4. Immerse frozen sections in Coplin jar and incubate at room temperature for 24 hr.
5. Transfer sections into Coplin jar containing a cobalt-formalin solution. Allow sections to undergo chelation-fixation for 1 hr.
6. Wash in running tap water for 5-10 min.
7. Mount in Glycerol-Gelatin.
8. Positive results of cytochrome oxidase are indicated by an almost black reaction.
9. Control slides are processed by the same procedure as test slides, but 10^{-3} M potassium cyanide is added to the reaction medium.

Atomic Absorption Determinations

Digestion Procedure

The surgical procedure for isolation of liver and kidneys consisted of positioning the embryo on a paraffin dish and opeing the abdominal area with a midline incision extending caudally. The liver

and kidneys were excised, placed in a dried preweighed acid cleaned test tube and the wet weight of the tissue was then recorded. If the tissue was not to be digested immediately it was frozen at 20°C until needed for analysis. After weighing, the tubes were placed in a beaker on a hot plate. Concentrated nitric acid (1 ml) was added and the sample was acid-digested for 12 hr. A second digestion was always necessary for complete digestion.

Preparation of Standards

A 4 ug/ml stock solution was prepared from commercially obtained reference standard of 1,000 ppm Cd. From this supply, fresh standard solutions of 2 ug/ml were prepared before each measurement. Standards were analyzed at the beginning and end of each run. A standard containing 2 ug/ml Cd will typically give an absorbance reading of about 0.3 absorbancy units.

Analysis

After digestion, the sample solutions were cooled and adjusted to a volume of 3 ml with deionized water. In order to avoid losses of metal by surface absorption, the samples were analyzed the same day by flame atomic absorption spectrophotometry (Model 107, Perkin-Elmer Corp.).

CHAPTER IV

EXPERIMENTAL OBSERVATIONS

The results obtained from exposing embryos to cadmium will be reported under general, histological and histochemical observations. A quantitative determination of cadmium in the kidneys and liver of these embryos by flame atomic absorption spectrophotometry is also included.

General Observations

Chicken embryos incubated for 48 hr were injected in ovo with 0.25 cc of cadmium chloride ($CdCl_2$) per egg and examined after further incubation (4-7 days). The survival rate of the experimental embryos was 68% (630/927) and that of controls was 87% (263/303). Hemorrhage appeared along the periphery of the cranial lobes and optic cups as well as along the neck. The embryos appeared to have acute peritonitis with a blood stained effusion.

Rupture of the visceral wall was another distinct observation in experimental embryos. In most cases following the rupture, visceral organs protruded and hemorrhaging was noted. Tables 1 and 2 summarize the mortality rate and per cent malformations resulting from exposure to cadmium and selenium, respectively.

Histological Observations

The normal architecture of the kidneys was altered by irregular areas of tubular destruction. This included tubular atrophy, with

Table 1. Effects of exposure of 48 hr chicken embryos to 0.25 cc of 0.025 M $CdCl_2$ at various stages of development.

Group	Number of injections	Age of embryo examined	Number-% viable embryos	% Malformed
Control*	42	4-day	32 (76%)	None
	39	5-day	29 (74%)	None
	119	6-day	100 (84%)	None
	135	7-day	102 (76%)	None
Experimental	119	4-day	88 (74%)	48%
	145	5-day	82 (56%)	52%
	466	6-day	221 (47%)	64%
	468	7-day	239 (51%)	72%

*Controls received 0.25 cc of 0.85% saline.

Table 2. Effects of exposure of 48 hr chicken embryos to 0.25 cc of 0.025 M Na-selenite at various stages of development.

Group	Number injected	Age of embryo examined	Number-% viable embryos	% Malformed
Control*	8	4-day	7 (88%)	None
	14	5-day	10 (71%)	None
	16	6-day	11 (68%)	None
	24	7-day	16 (67%)	None
Experimental	18	4-day	15 (83%)	30%
	30	5-day	22 (73%)	32%
	34	6-day	23 (68%)	40%
	36	7-day	23 (64%)	45%

*Controls received 0.25 cc of 0.85% saline.

tubular remnants lying within increased interstitial tissue containing an abundance of chronic inflammatory cells. Occasionally, tubules were dilated with flattened lining cells and contained erythrocyte material (Figs. 1-2). In some instances, a generalized hemorrhagic condition predominated on one side of the embryo (Fig. 3).

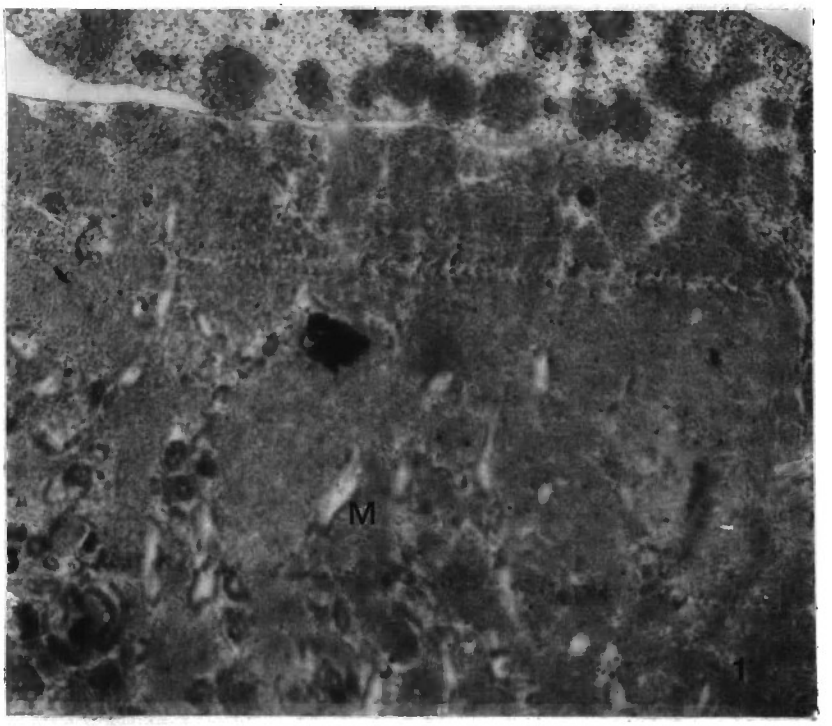
Four and five-day embryos were not as susceptible to cadmium as the six and seven-day ones. Although there were no drastic changes at earlier stages of development, slight swelling of the epithelial cells of mesonephric tubules and ruptured blood vessels were recognized upon histological examination (Figs. 4-5). However, the lesions were most marked in the six and seven-day-old embryos, in which there was exfoliation of tubular epithelial cells with many being desquamated and the remainder granulated and partly destroyed (Figs. 6-7). A certain reduction in the number of nuclei in the tubuli and a shedding of cell nuclei towards the tubular lumen was also detected. In some instances the greater part of the tubular structure was effaced and only the tubular wall seemed to remain (Figs. 8-9).

Marked degeneration was also observed in the glomeruli. Necrosis and partial hyalinization were seen in the glomerular capillaries, and adhesions were occasionally observed between Bowman's capsule and glomerular capillaries (Fig. 10). Associated with the rupturing of the visceral wall were hematomas and a profusion of red blood cells where the wall separated from the visceral organs (Fig. 11).

The lesions were not limited to the proximal tubules but were

Fig. 1. Cross section of cadmium-treated embryo showing a profusion of red blood cells and edema of mesonephros (m). 20X

Fig. 2. Cross section of cadmium-treated embryo showing tubular atrophy, with tubular remnants lying within increased interstial tissue (arrows). 20X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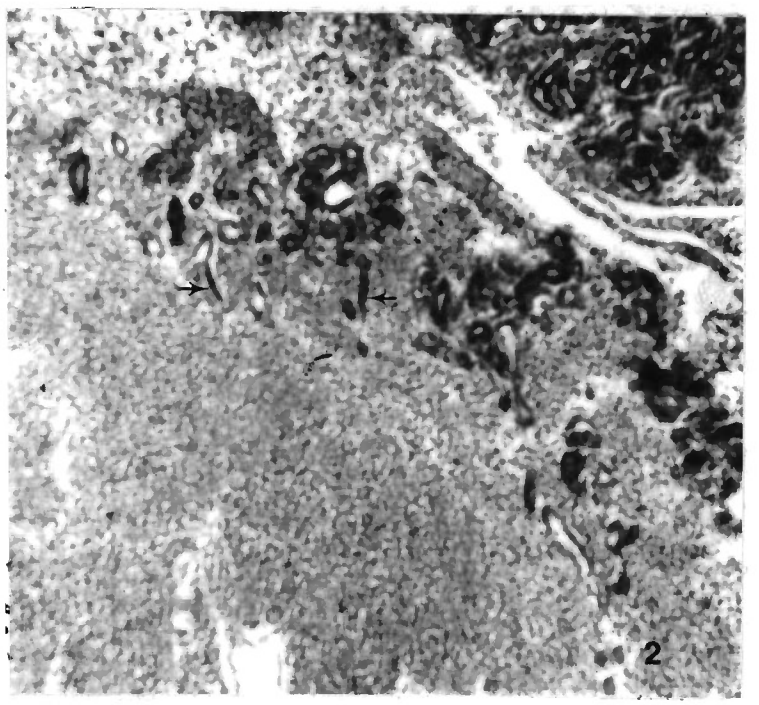
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Fig. 3. Overview of cadmium-treated embryo showing a generalized hemorrhagic condition predominating on one side (arrow). 4X

Fig. 4. Cross section of cadmium-treated embryo showing a slight swelling of the epithelium and hemorrhaging (h) in the mesonephros (arrow). 10X

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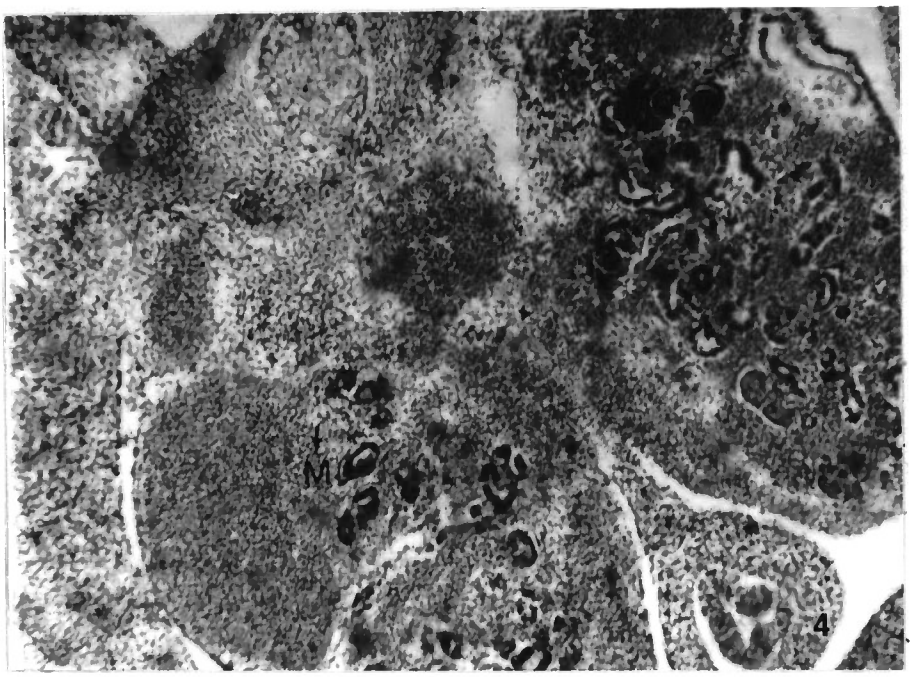

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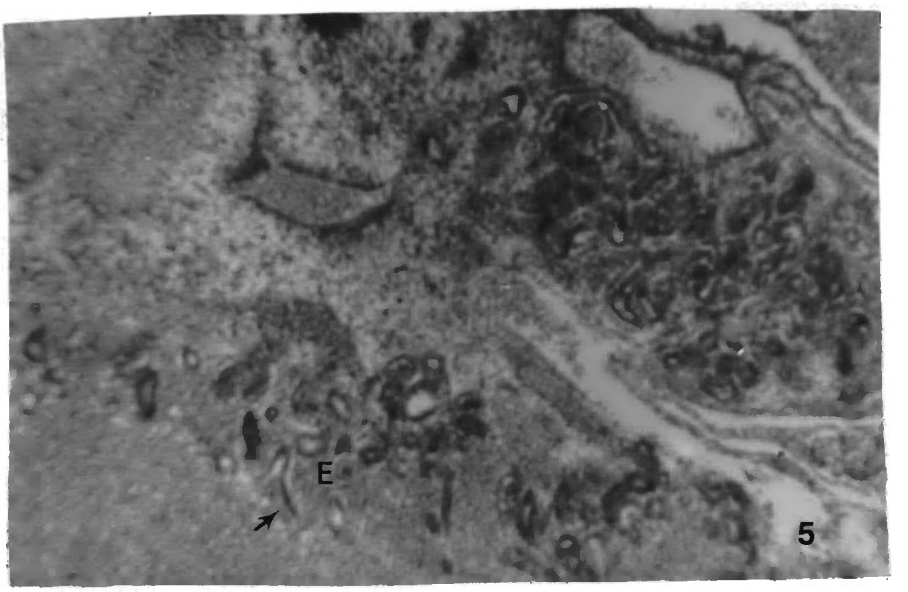
Fig. 5. Cross section of cadmium-treated embryo showing unilateral hemorrhage (arrow) and edematous (e) condition of the mesonephros. 10X

Fig. 6. Sagittal section of cadmium-treated embryo showing partial destruction of mesonephric tubular membranes (arrow). 45X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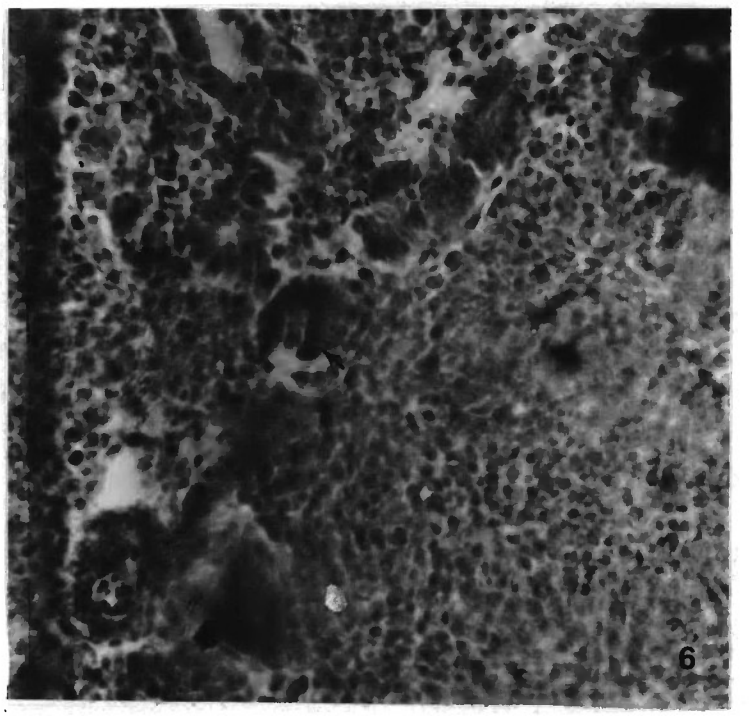
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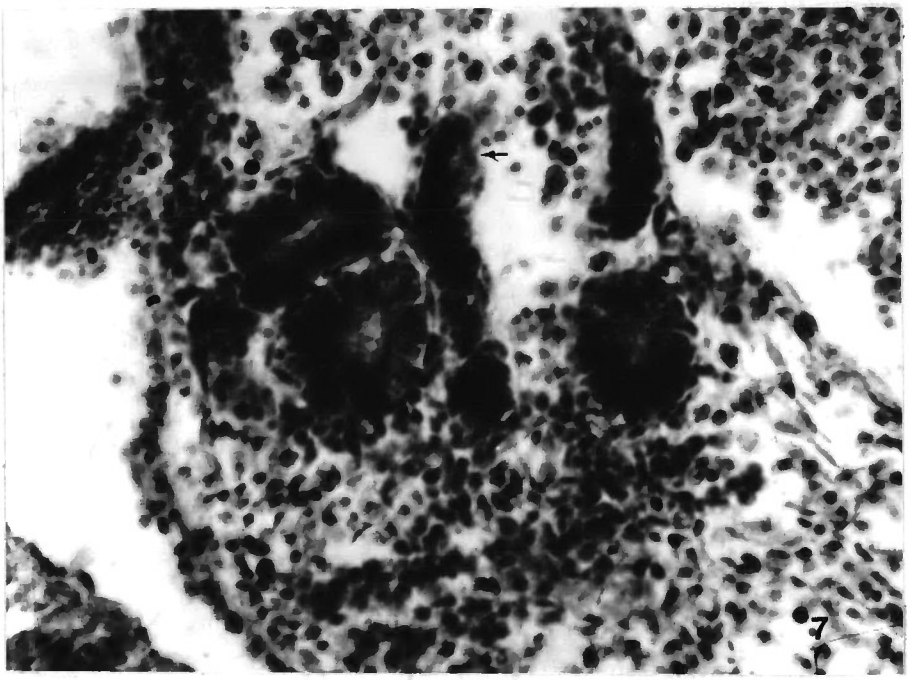
Fig. 7. Cross section of cadmium-treated embryo showing exfoliation of mesonephric tubular cells (arrow). 45X

Fig. 8. Higher magnification of cadmium-treated mesonephric tubule showing shedding of cell nuclei (n) towards the tubular lumen (arrow). 100X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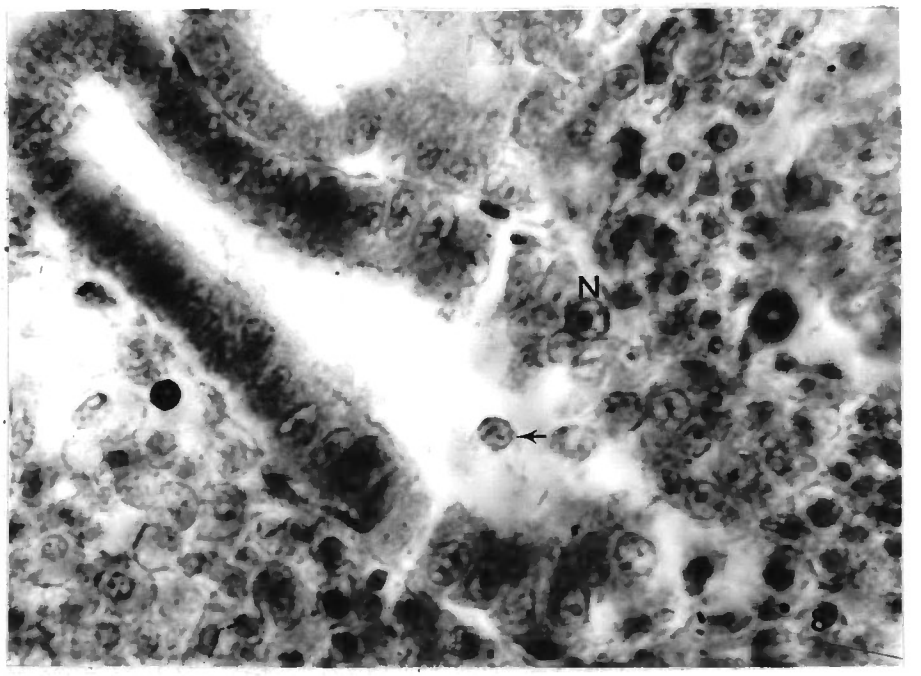
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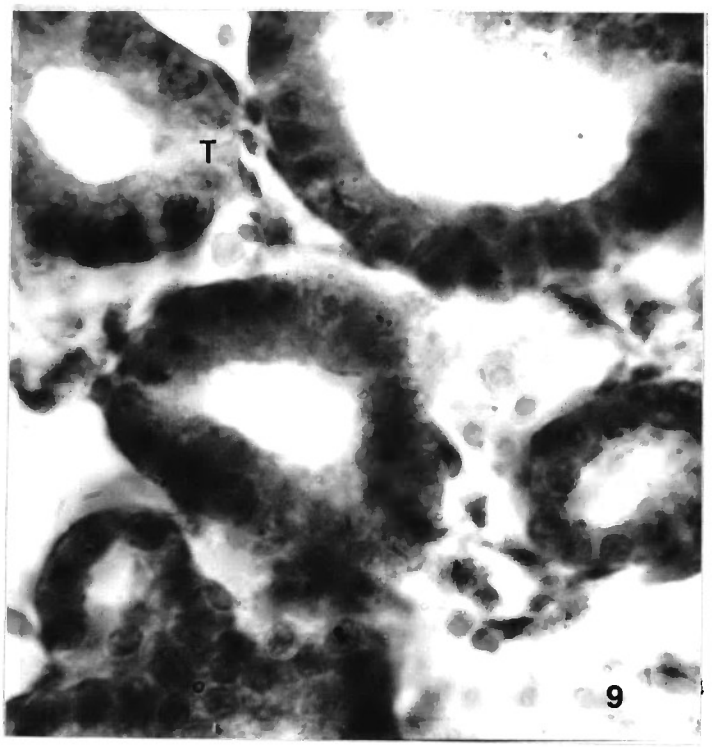
Fig. 9. Cross section of cadmium-treated embryo showing mesonephros having a portion of the tubular(t) structure effaced. 100X

Fig. 10. Cross section of cadmium-treated embryo showing marked degeneration in the glomerulus (g). Note adhesions between Bowman's capsule and glomerulus (arrow). 100X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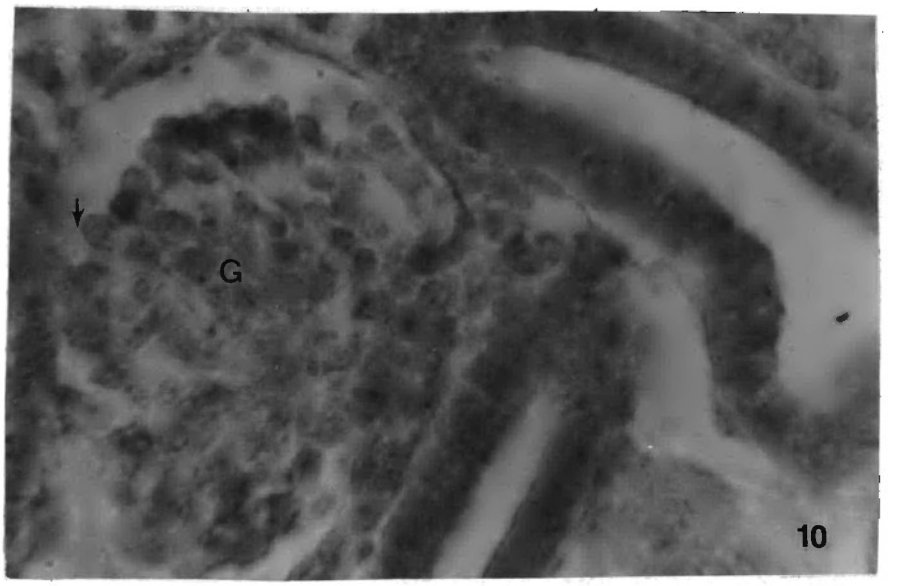
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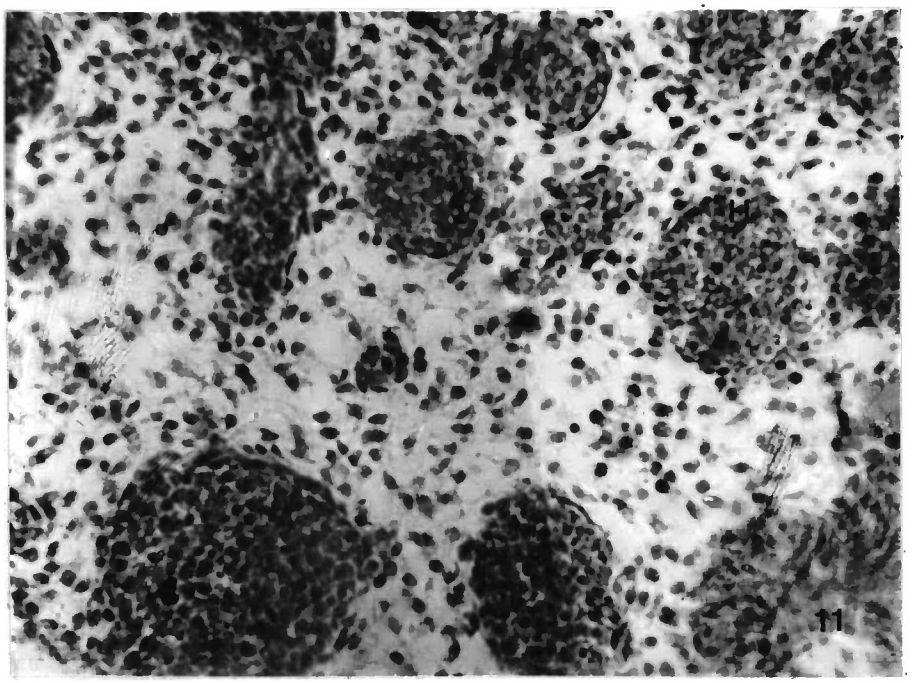
Fig. 11. Cross section of cadmium-treated embryo showing hematomas (h) in the visceral wall and a profusion of red blood cells. 45X

Fig. 12. Cross section of cadmium-treated embryo showing necrotic lesions distributed throughout the liver (arrow). 10X



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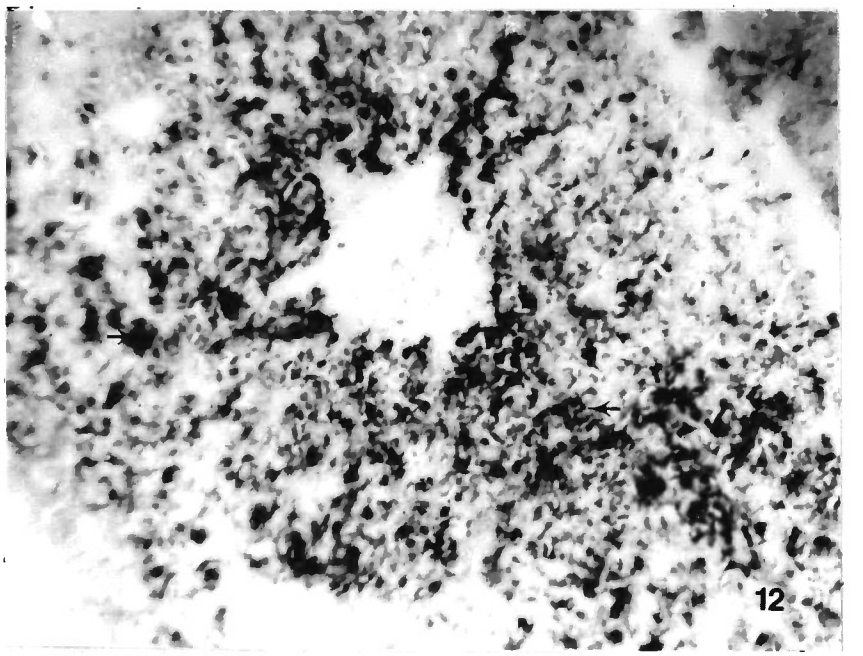

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found to a lesser degree in other organs. Necrotic lesions were irregularly distributed throughout the liver. Their outlines were somewhat defined and situated at the midzone of the tissue. Blood cysts, areas of destruction of the parenchyma filled with recently extravasted erythrocysts, were common (Figs. 12-13). A study of sections from selenium-treated embryos showed the greater part of the tubular epithelium disarranged and a mass of red blood cells was detected in the mesonephros. However, destruction in the majority of selenium-treated embryos (Figs. 14-15) was not as great as that of cadmium. In controls, the mesonephric tubular epithelial cells were well preserved without remarkable changes. Hepatic tissue from control embryos displayed no evidence of morphological alterations (Figs. 16-18).

Histochemical Observations

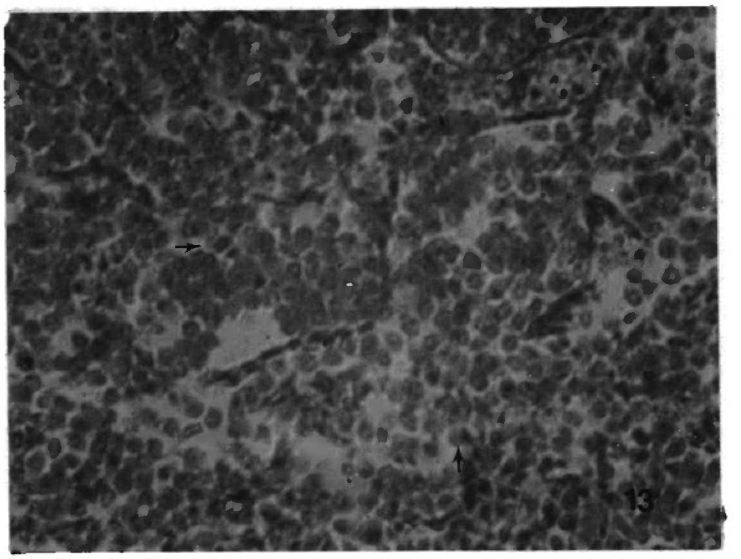
Acid Phosphatase

With the Naphthol AS-MX phosphate method of Kaplow and Burstone (1962) for acid phosphatase, enzymatic activity was found to be present in the kidneys of control as well as experimental embryos. In kidneys from control embryos faint spotting of acid phosphatase activity was noted among the mesonephric tubules with increasing activity in the most proximal portion (Figs. 19-20).

In five-day post-incubation kidneys, the overall amount of acid phosphatase activity in the lesion was much reduced by comparison with the seven-day old specimens. The acid phosphatase positive plugs

Fig. 13. Cross section of cadmium-treated embryo showing areas of destruction of the parenchyma filled with ruptured blood vessels (arrows). 45X

Fig. 14. Section of a selenium-treated embryo showing most mesonephric tubules (t) destroyed or disarranged (arrow). 20X



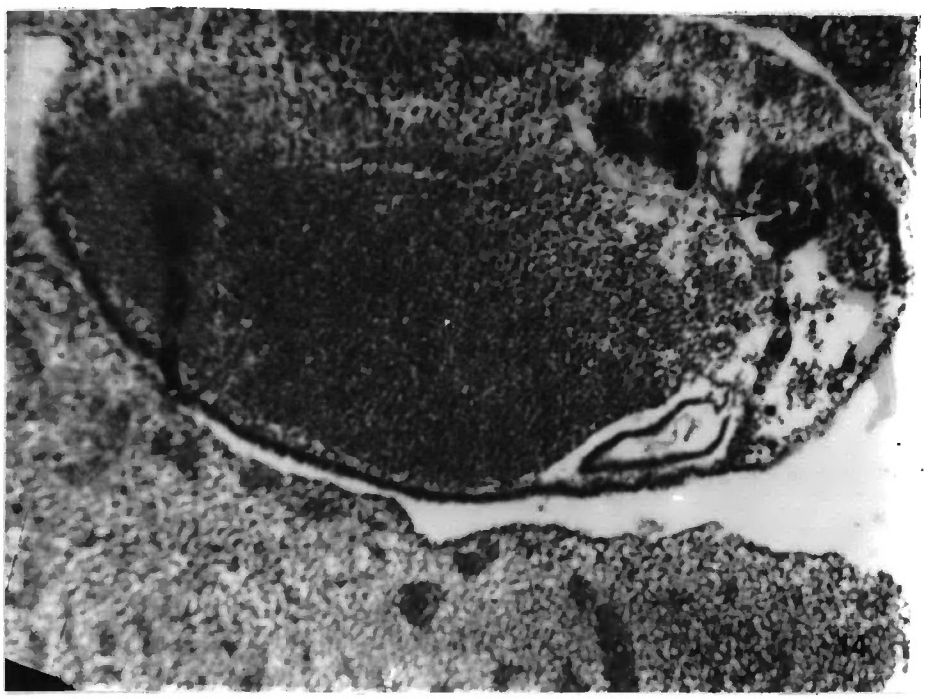
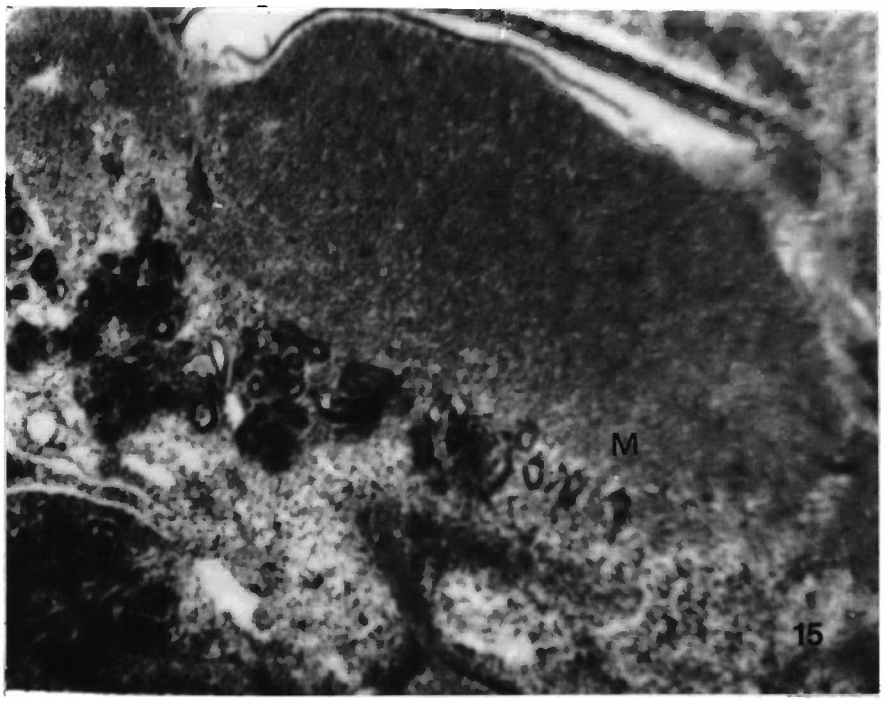


Fig. 15. Cross section of selenium-treated embryo showing a mass of ruptured blood vessels in the mesonephros (m). 20X

Fig. 16. Sagittal section of control embryo. Note the well preserved glomerulus (g) and absence of swelling in mesonephros (arrow). 20X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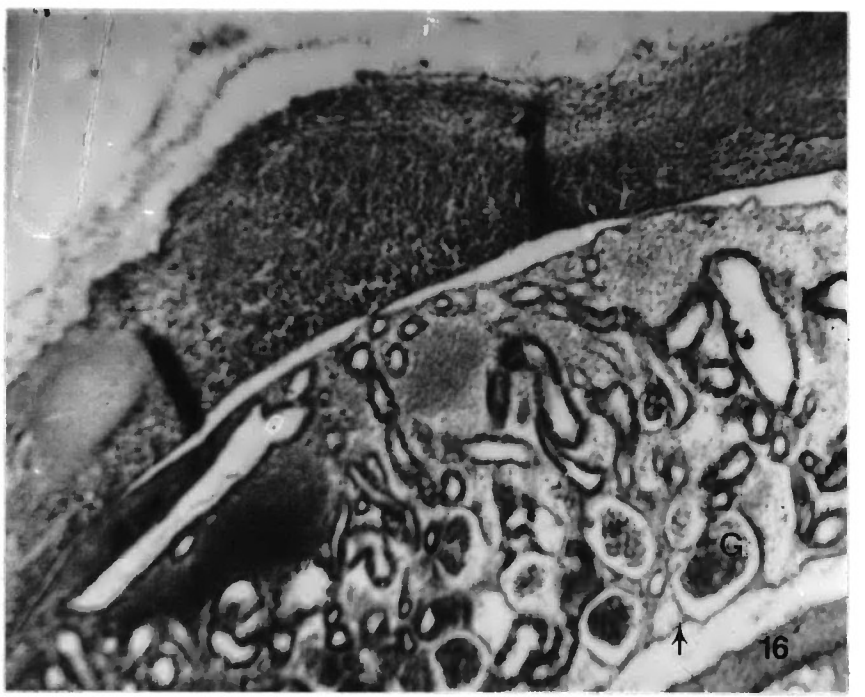
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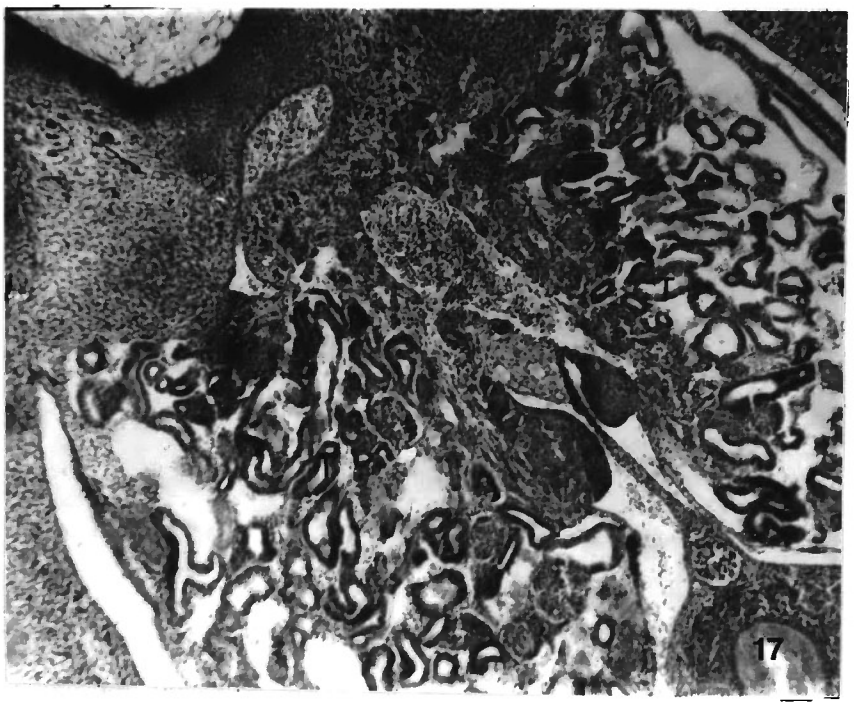
Fig. 17. Cross section of control embryo showing intact tubular (t) structures and absence of ruptured blood vessels. 10X

Fig. 18. Cross section of control embryo showing no evidence of morphological alterations in the hepatic tissue. Note, however, the few ruptured blood vessels (arrow). 45X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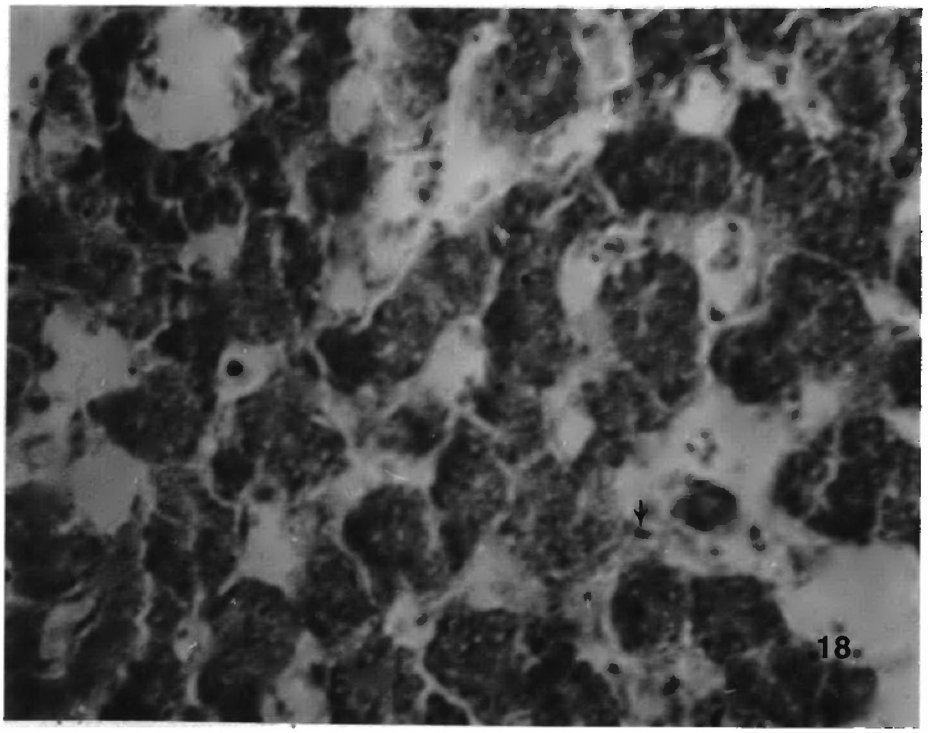
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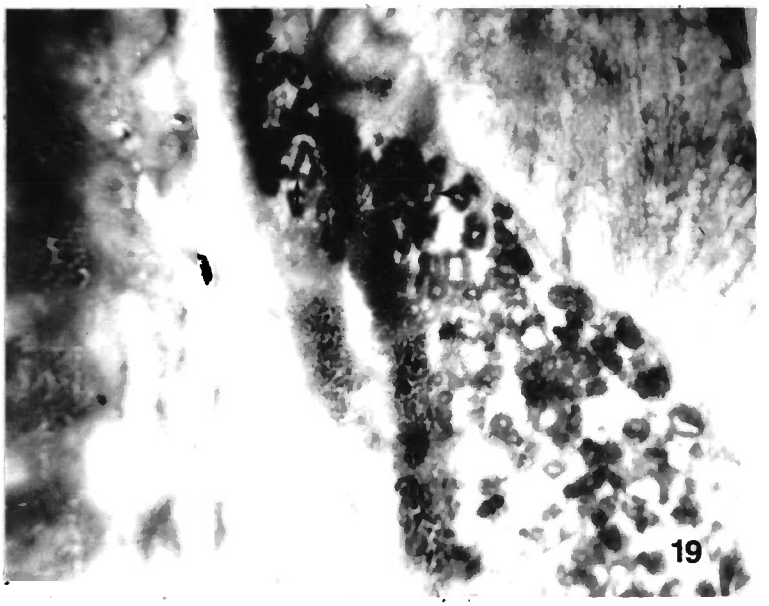
Fig. 19. Sagittal section of control embryo showing increase of acid phosphatase activity proximally in mesonephros (arrow). 10X

Fig. 20. Sagittal section of control embryo showing faint spotting of acid phosphatase activity (arrow). 20X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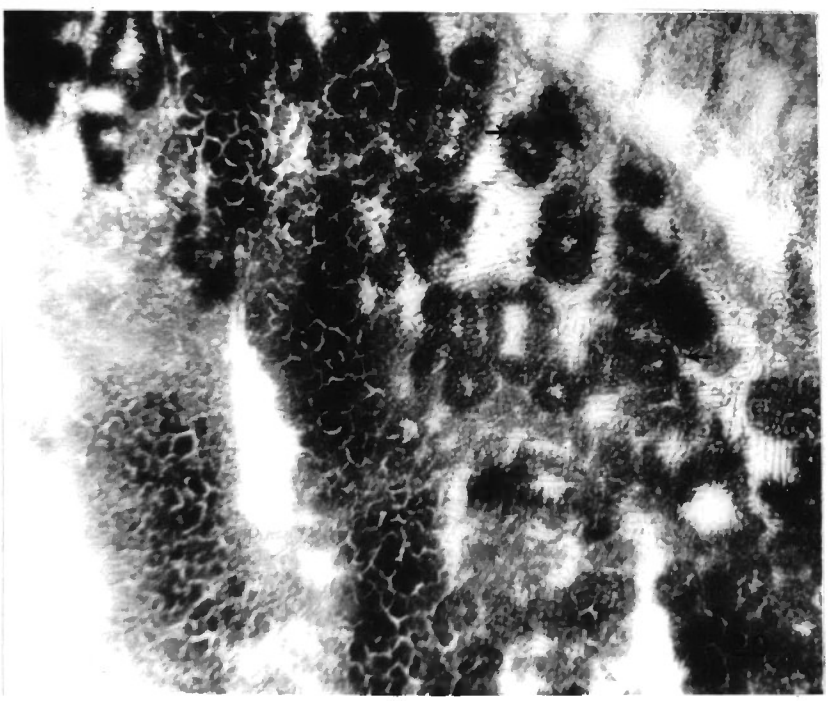

filling the tubules were no longer seen, but isolated concentrations were still present in the lumen of the damaged tubules (Fig. 21).

At six-day post-incubation, the acid phosphatase activity had attained a disarrayed distribution, in that areas of intense activity were isolated as islands surrounded by regions in which the reaction was either very weak or negative. Tubules could be observed in which only some of the peripheral cells were positive for this enzyme while other cells immediately adjacent appeared negative. Some tubules gave an intense, total cytoplasmic reaction, while others closely associated with them, presented only a minimal, stippled granular reaction. Glomeruli in this boundary region were filled with masses of homogeneous material, with a few discrete positively stained granules therein (Figs. 22-23).

At seven-day post-incubation, the amount of acid phosphatase activity appeared to show an increase over the five and six-day kidney. In many instances the lumen of tubules filled with acid phosphatase positive material were seen. In such tubules there was no delineation of cells from each other in the tubular epithelium, indicating either loss of nuclear components or terminal stages of degeneration (Figs. 24-27).

In the contralateral kidneys of cadmium-treated embryos the distribution of acid phosphatase activity appeared to be almost the same as that seen in the control embryos. Here the reaction was demonstrated as fine, granular material in the epithelium of distal tubules (Fig. 28). Acid phosphatase activity in the liver was demonstrated as fine, granular

Fig. 21. Cross section of cadmium-treated embryo showing isolated areas of acid phosphatase material (arrows). 20X

Fig. 22. Sagittal section of cadmium-treated embryo showing intense acid phosphatase activity in some regions of the mesonephros (m). Note other areas closely associated presenting only a stippled granular reaction (arrow). 20X


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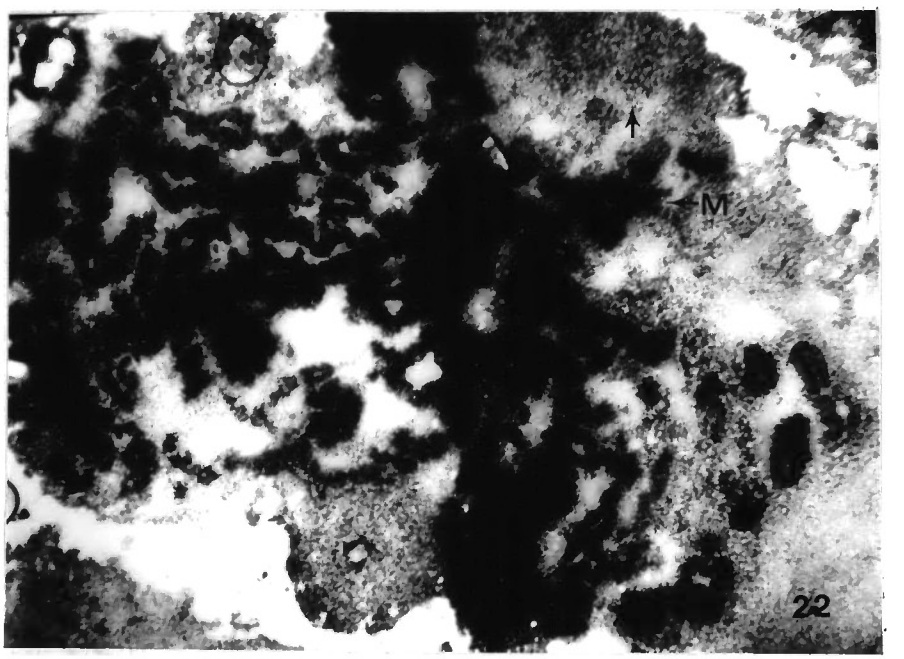
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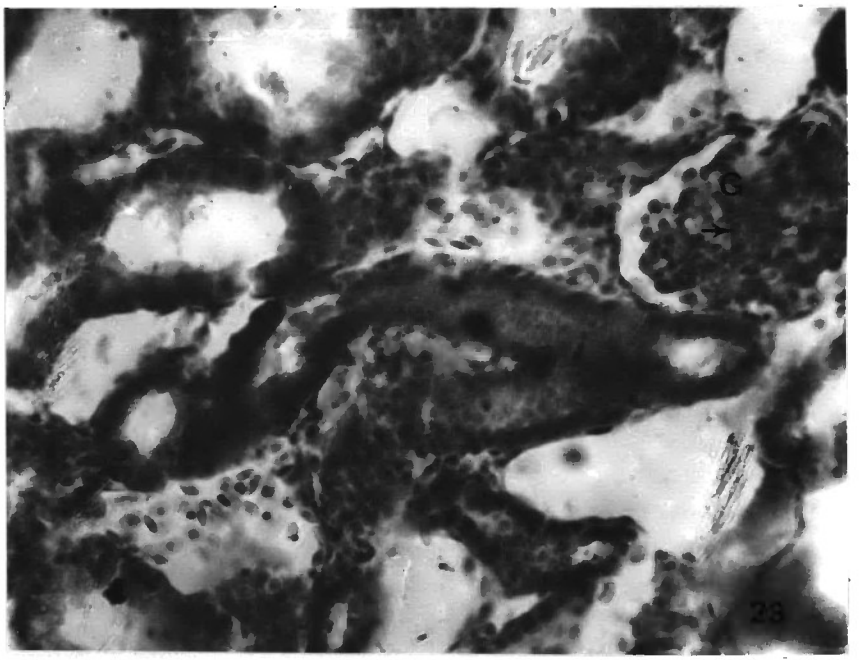
Fig. 23. Higher magnification of cadmium-treated embryo showing glomerulus (g) filled with a few discrete positively stained granules (arrow). 45X

Fig. 24. Sagittal section of 7-day cadmium-treated embryo showing an increase in acid phosphatase activity among the tubules (arrow). 45X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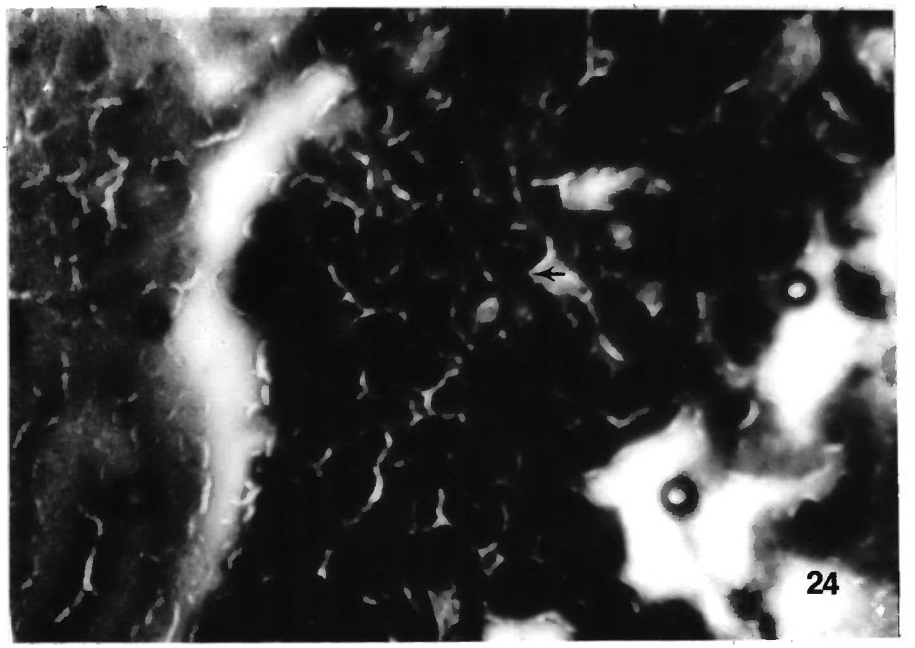
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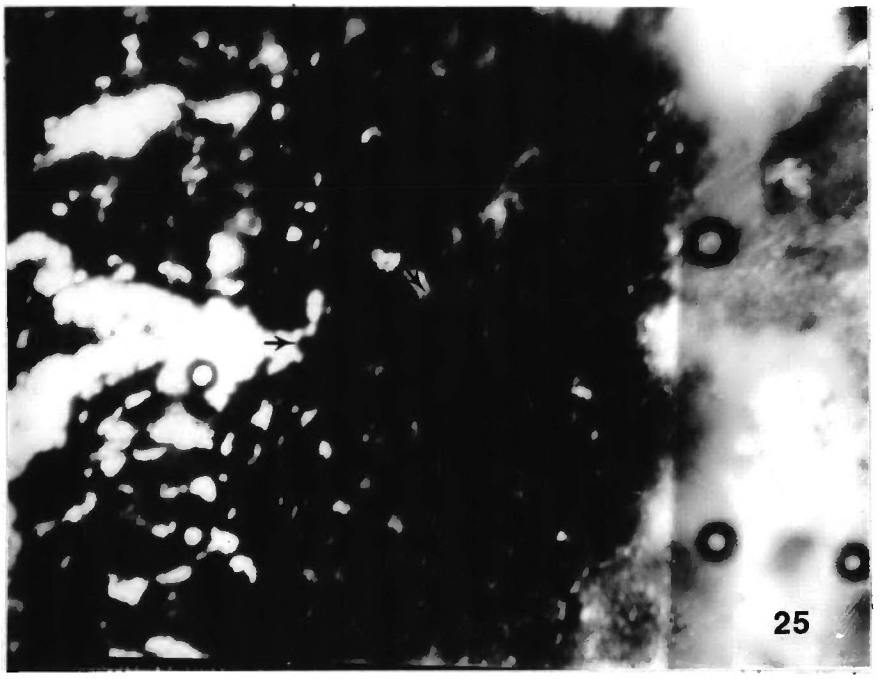
Fig. 25. Cross section of 7-day cadmium-treated embryo. Note the intensity of stain such that the outlining of tubules is lost (arrows). 45X

Fig. 26. Higher magnification of tubular area showing the presence of acid phosphatase activity along the tubular membrane (arrow). 100X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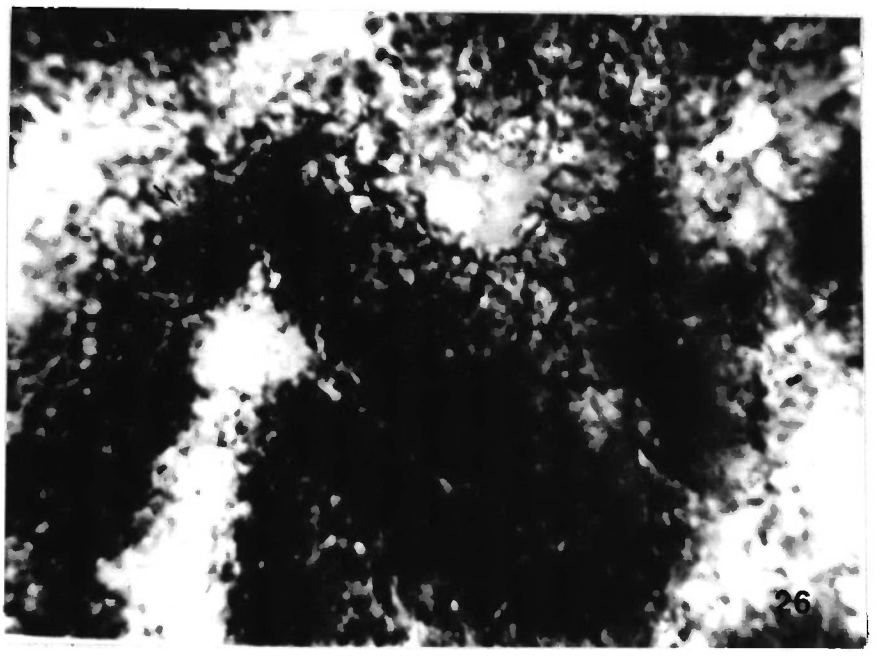
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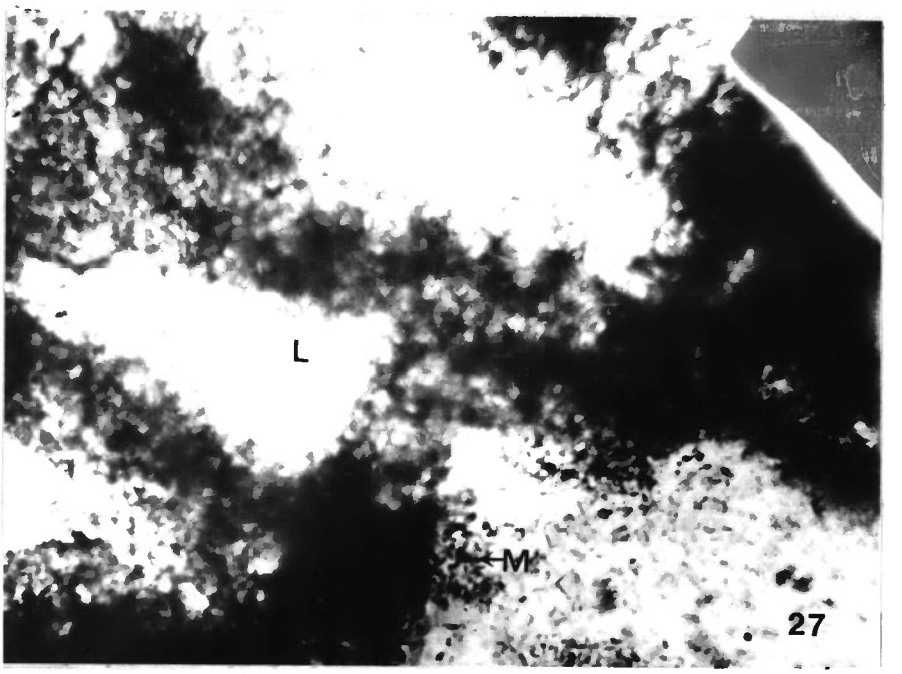
Fig. 27. Higher magnification of tubules showing heavy deposits of acid phosphatase along the tubular membranes (m). Note the absence of activity within the lumen (l). 100X

Fig. 28. Sagittal section showing contralateral kidney of cadmium-treated embryo (c). Note the scattered deposits of acid phosphatase, similar to the distribution in control embryo (arrow). 10X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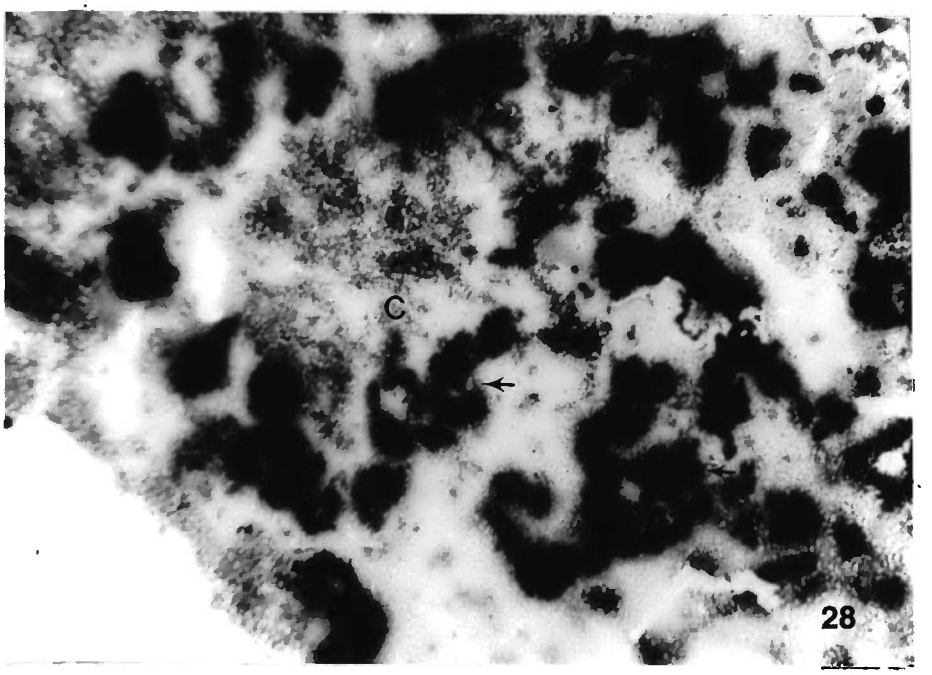
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material disperse among the disorderly parenchyma cells, with a light to moderate reaction in the control tissue (Figs. 29-30). Control slides appeared extremely faint and showed no signs of enzymatic activity (Fig. 31).

Alkaline Phosphatase

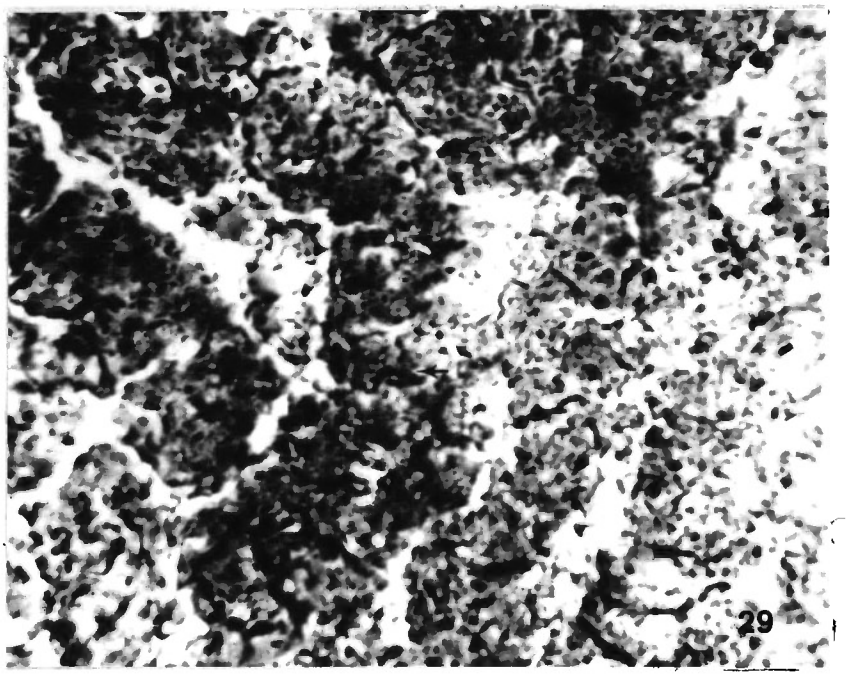
In seven-day cadmium-treated embryos, a discrete finely granular reaction was present in the epithelial cytoplasm of both proximal and distal tubules. Apart from isolated spots of activity, the tubules were negative for alkaline phosphatase, although tubules positive for alkaline phosphatase were present immediately adjacent to it (Fig. 32).

At six days post-incubation, condensed regions in which alkaline phosphatase activity was present as a finely granular, evenly dispersed deposits were observed. The mesonephros at this stage had a more intense alkaline phosphatase reaction than was seen at seven days post-incubation. The finely granular deposits of alkaline phosphatase activity were present in both the proximal and distal areas, with the heavier staining exhibited in the proximal tubules (Fig. 33).

Specimens of contralateral kidneys presented an appearance which was similar in all respects to that found in the control kidneys (Fig. 34). The proximal and distal tubules in the control kidneys demonstrated a much more intense reaction of alkaline phosphatase than in any stage of experimental (cadmium-treated) embryos. The activity was in the majority of cases so intense that the lumen of the tubules was almost completely obscured (Figs. 35-36). The reaction in the liver

Fig. 29. Cross section of cadmium-treated embryo showing granular deposits of acid phosphatase among disorderly parenchyma cells (arrow). 45X

Fig. 30. Cross section of control embryo showing light to moderate acid phosphatase reaction products in liver (arrows). 45X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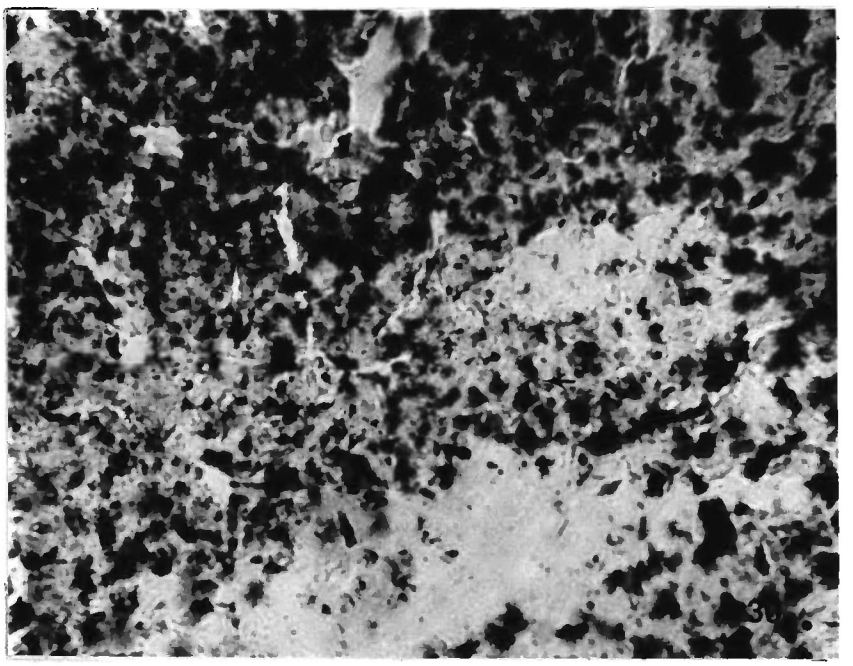

Fig. 31. Photograph of control slide showing faint outlines of cells (arrow) but no signs of enzymatic activity. 10X

Fig. 32. Sagittal section of 7-day cadmium-treated embryo showing discrete, isolated deposits of alkaline phosphatase among tubules (arrows). 10X

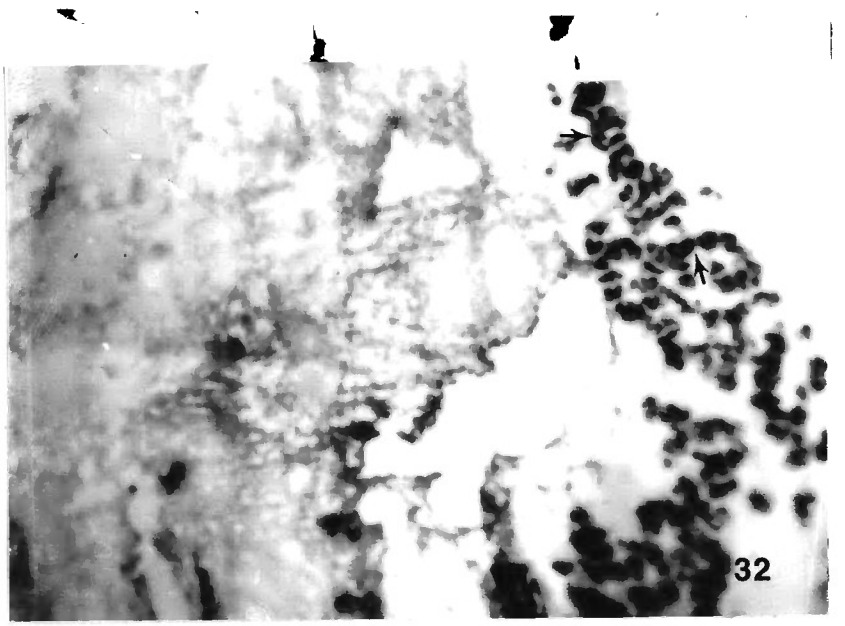
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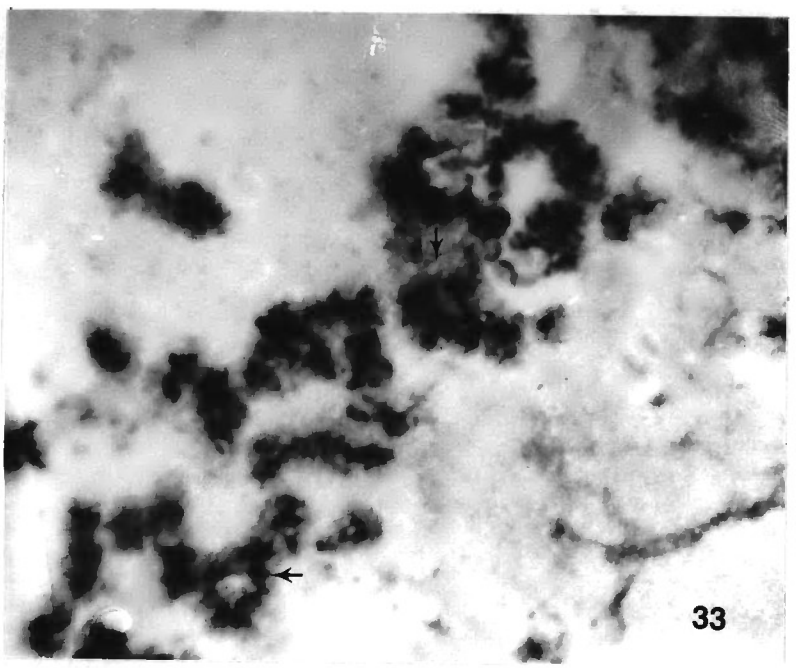
Fig. 33. Sagittal section of 6-day cadmium-treated embryo. Note that the disrupted mesonephros at this stage had more intense alkaline phosphatase reaction products than was seen at 7-day (arrows). 20X

Fig. 34. Cross section of cadmium-treated embryo. Note that contralateral kidney(c) showed heavier deposits of alkaline phosphatase (arrows). 20X



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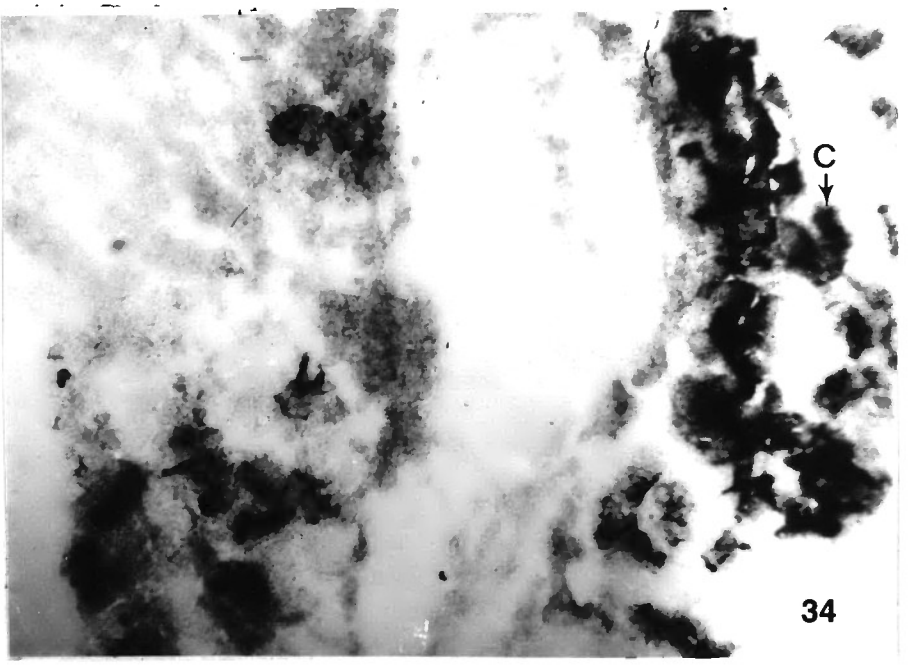

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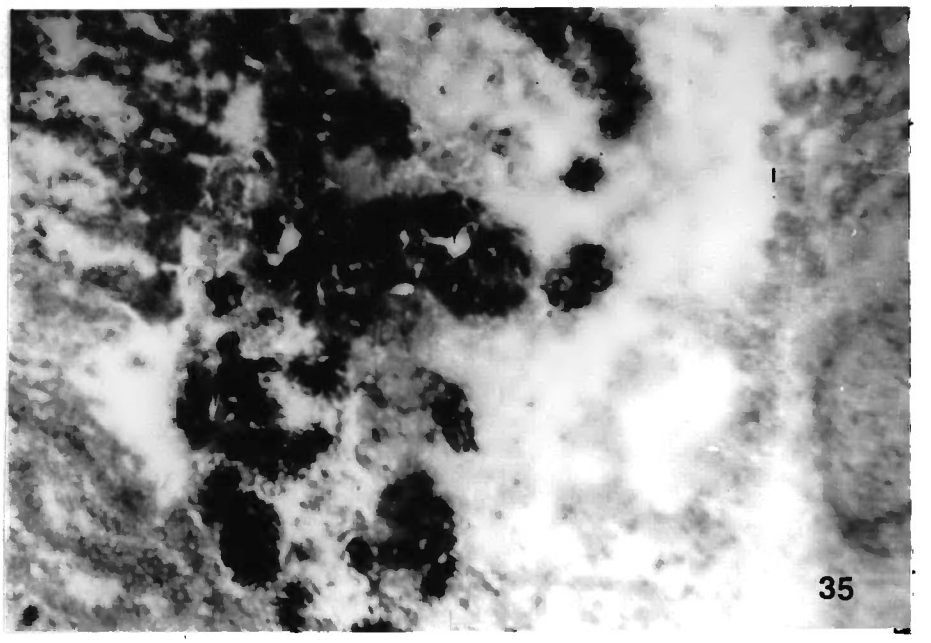
Fig. 35. Sagittal section of control embryo showing alkaline phosphatase activity so intense that the lumen of tubules was almost completely obscured (arrows). 20X

Fig. 36. Higher magnification of control embryo. 45X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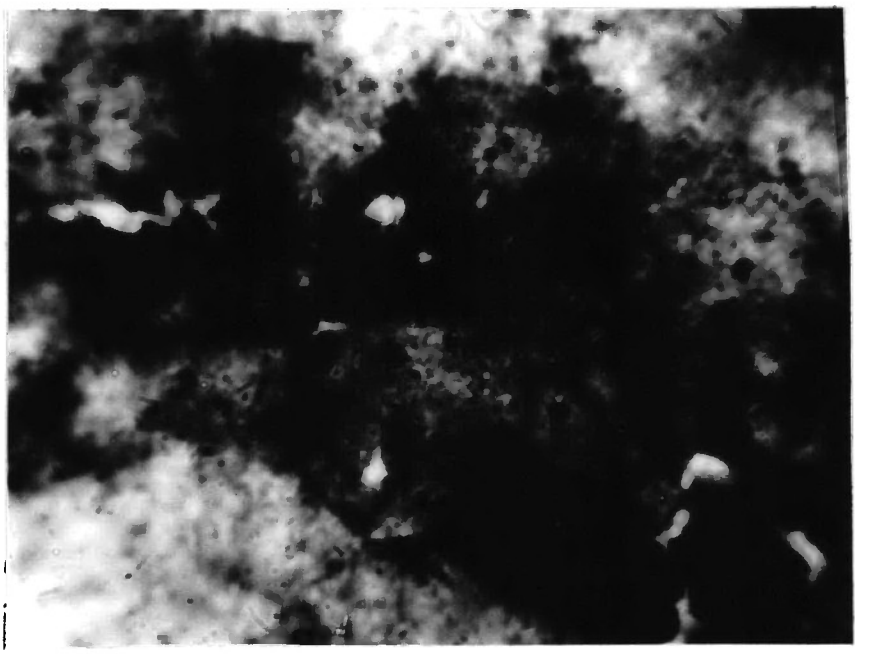


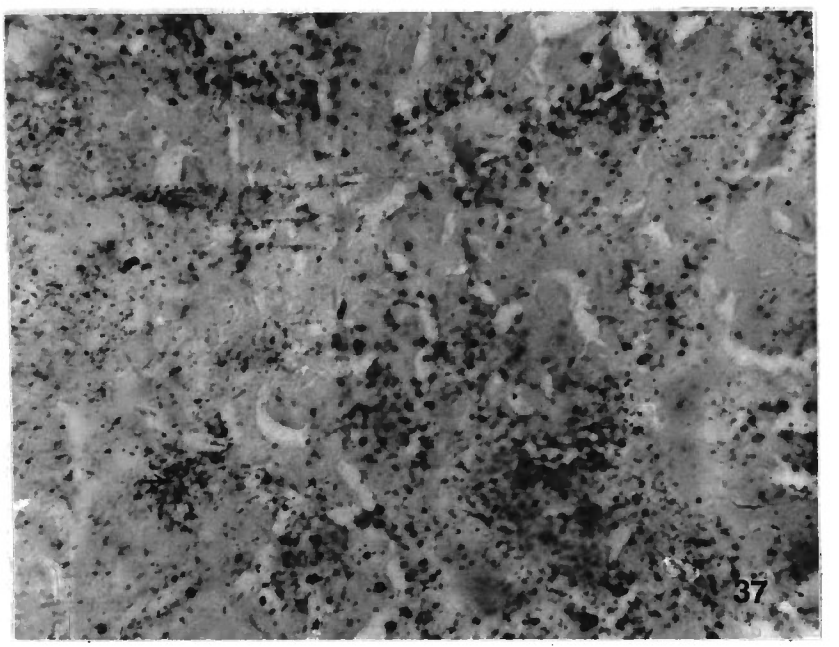
was spread out along the membranes of parenchyma cells (Fig. 37). While the control liver showed aggregates of alkaline phosphatase activity among cells (Fig. 38). Sections incubated in an incubation mixture lacking Naphtol Alkaline phosphatase never showed deposits of alkaline phosphatase activity (Fig. 39).

Beta-Glucuronidase

Beta-glucurondiase activity visualized as bluish-green granules was detected in kidney tubules of cadmium-treated embryos. Tubular epithelium gave a slightly positive reaction for beta-glucuronidase activity, becoming progressively stronger toward the lumen of the tubule. The kidney revealed a strong cytoplasmic reaction in both proximal and distal convoluted tubules as well as brush-border staining. Occasionally some mesonephric tubules were negative for brush-border reaction, while others immediately adjacent demonstrated a strong brush-border reaction. The nephrogenic cells at the periphery of the kidney sections stained a spotted bluish-green for localization of beta-glucuronidase. The beta-glucuronidase activity shifted to the cytoplasmic droplets of the cells (Fig. 40) and appeared to be dense aggregates. Kidney tubules at sites of damaged tissue gave an intense reaction, in some instances, with material filling the lumen of the tubules. In almost all cases the reaction product was heavily deposited in the interstices of the tubules. The finely granular reaction that was obtained differed from acid phosphatase reactions in that a more discrete type reaction was seen in the tubules with the acid phosphatase stain. The mesonephric tubules in the

Fig. 37. Cross section of cadmium-treated embryo showing dispersed deposits of alkaline phosphatase among hepatic tissue (arrows). 45X

Fig. 38. Cross section of control embryo showing aggregates of alkaline phosphatase reaction product among hepatic tissue (arrows). 45X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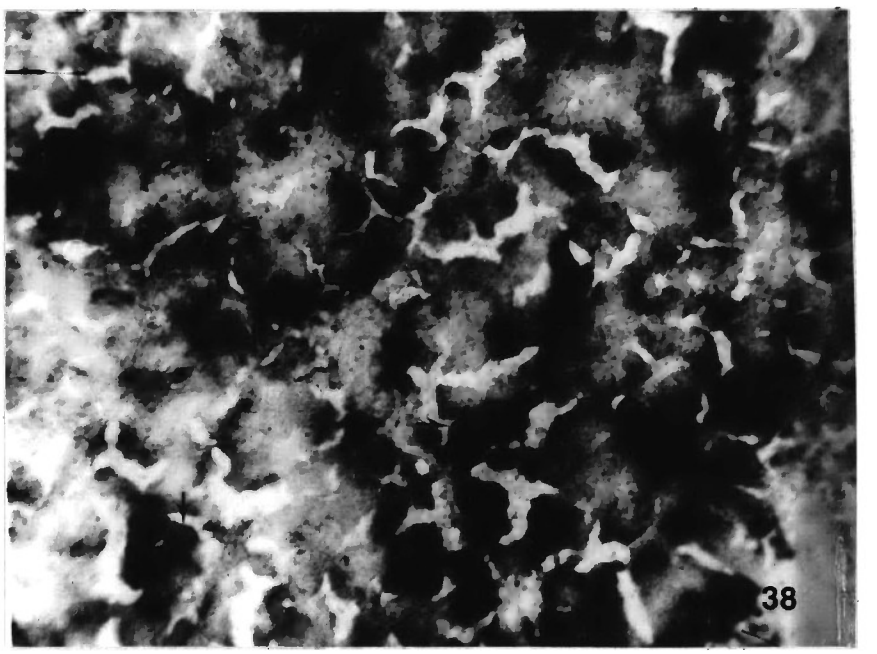
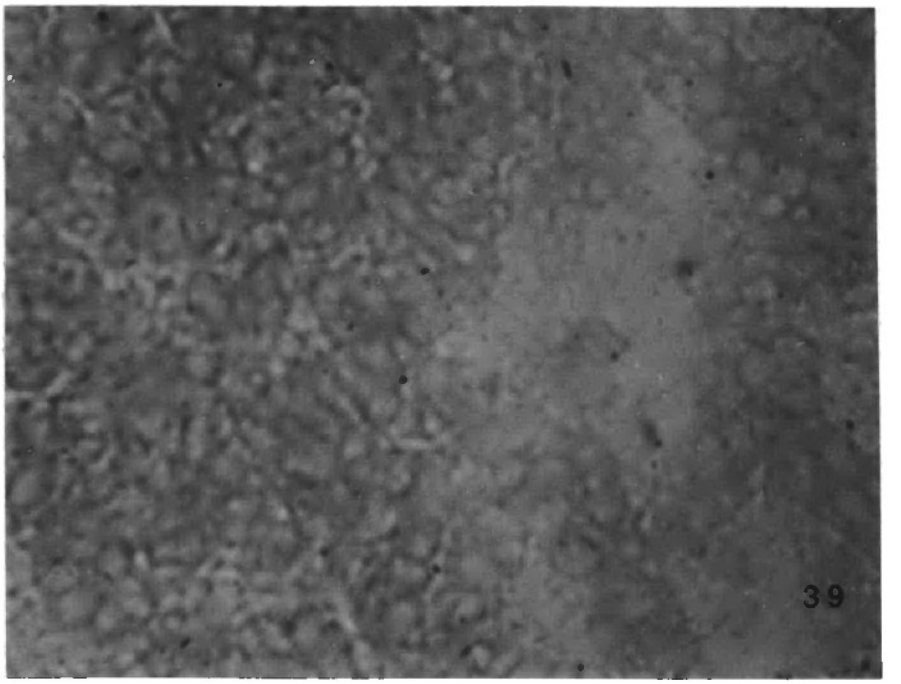

38

Fig. 39. Photograph of control slide showing no deposits of alkaline phosphatase reaction products. 10X

Fig. 40. Cross section of cadmium-treated embryo showing bluish-green stain, among mesonephric tubules, indicative of beta-glucuronidase activity. Note the stronger reaction products in lumen rather than in membranes (arrows). 45X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40

control kidneys demonstrated a stippled-like reaction of beta-glucuronidase. These granular specks were sparsely distributed through the lumen and epithelium of the tubules (Fig. 41). Incubating sections in a medium containing potassium hydrogen saccharate completely inhibited beta-glucuronidase activity (Fig. 42).

Cytochrome Oxidase

Both experimental and control kidneys showed positive results for cytochrome oxidase activity. However, as in the case of alkaline phosphatase, the cytochrome oxidase activity was more observable in the control sections than in the experimental. Sections from six-day control (saline-treated) embryos showed frequent enzyme positive granules in the membrane of tubular structures. Granular specks were seen within the tubular spaces (Fig. 43). A higher magnification of a 7-day mesonephric section demonstrates localization of cytochrome oxidase activity in the periphery of the tubular membrane. Very little activity is seen at the brush-border of the tubule (Fig. 44).

Liver tissue of control embryos showed a more intense staining of hepatic cells when compared to mesonephric tissue. Cytochrome oxidase deposits are so heavy that only a few areas are lacking the presence of stain (Fig. 45). However, experimental embryos are not as susceptible to cytochrome oxidase as controls. Hepatic tissue of experimental organisms is lightly deposited with stain, indicating an inhibitory effect similar to the situation with alkaline phosphatase (Fig. 46). Controls were prepared by incubating as for normal staining but with the addition of potassium cyanide to the reaction medium (Fig. 47).

Fig. 41. Sagittal section of control embryo showing stippled-like reaction products of beta-glucuronidase. Note the sparse distribution of granular specks among the tubules (arrows). 45X

Fig. 42. Photograph of control slide showing negative reaction for beta-glucuronidase. 45X


41

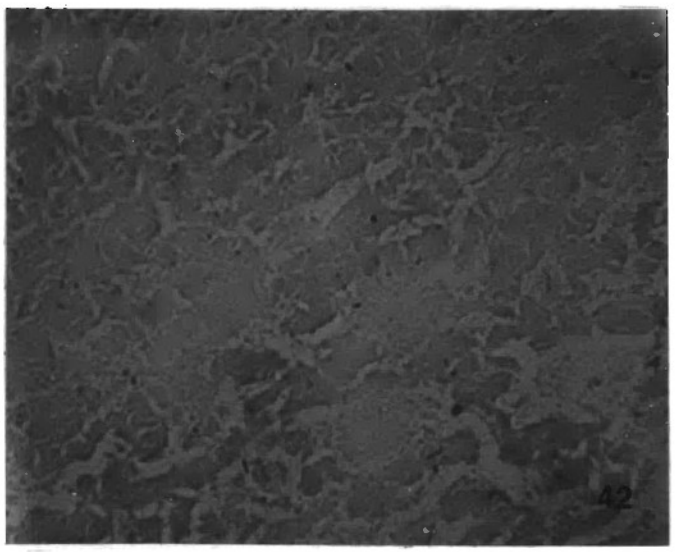
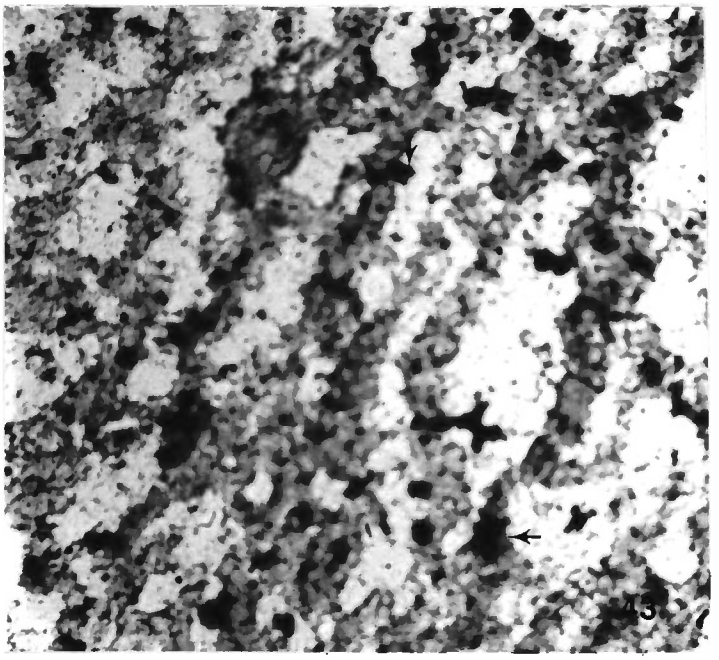

42

Fig. 43. Sagittal section of control embryo showing positive granules of cytochrome oxidase reaction product among tubular structures (arrows). 20X

Fig. 44. Higher magnification of mesonephric section showing localization of cytochrome oxidase reaction products in periphery of disrupted tubular membrane (m). 45X



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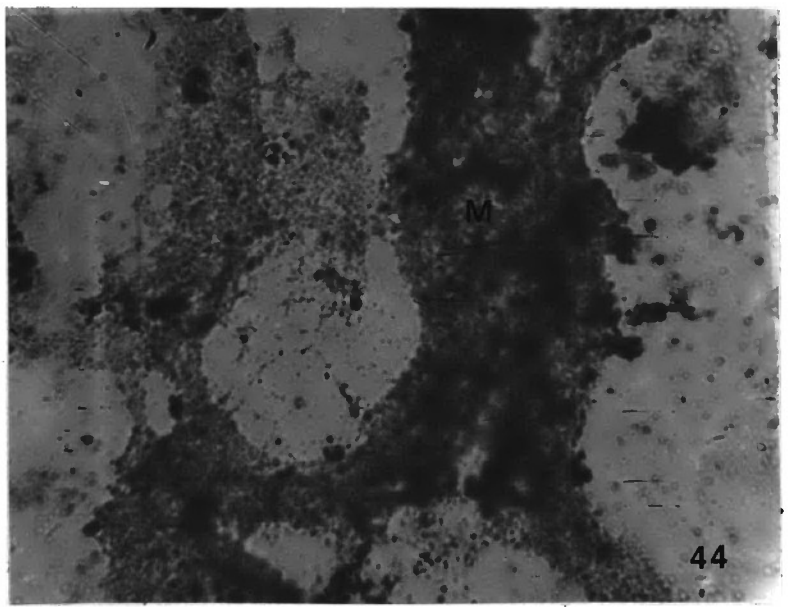
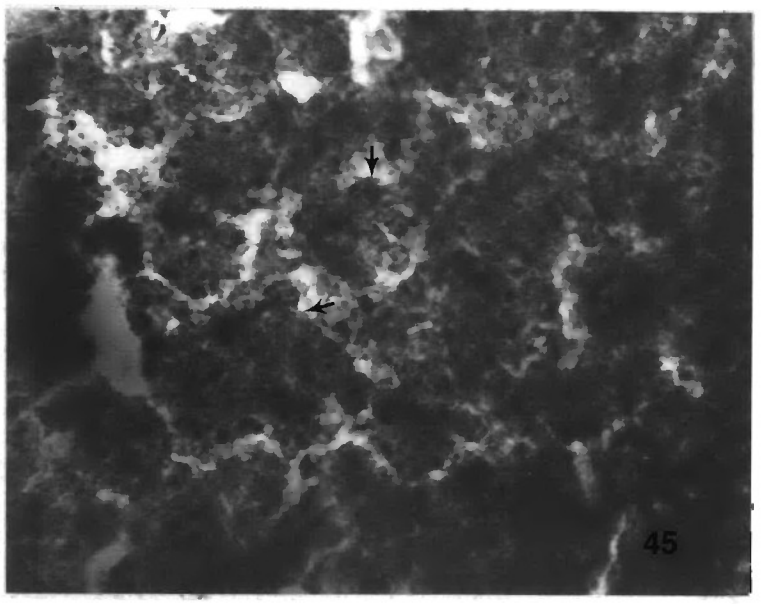


44

Fig. 45. Sagittal section of control embryo showing heavy deposits of cytochrome oxidase reaction products in liver tissue. Note the more intense staining of hepatic cells when compared to mesonephric tissue (arrows). 45X

Fig. 46. Sagittal section of cadmium-treated embryo showing only light deposits of cytochrome oxidase activity (arrows). 45X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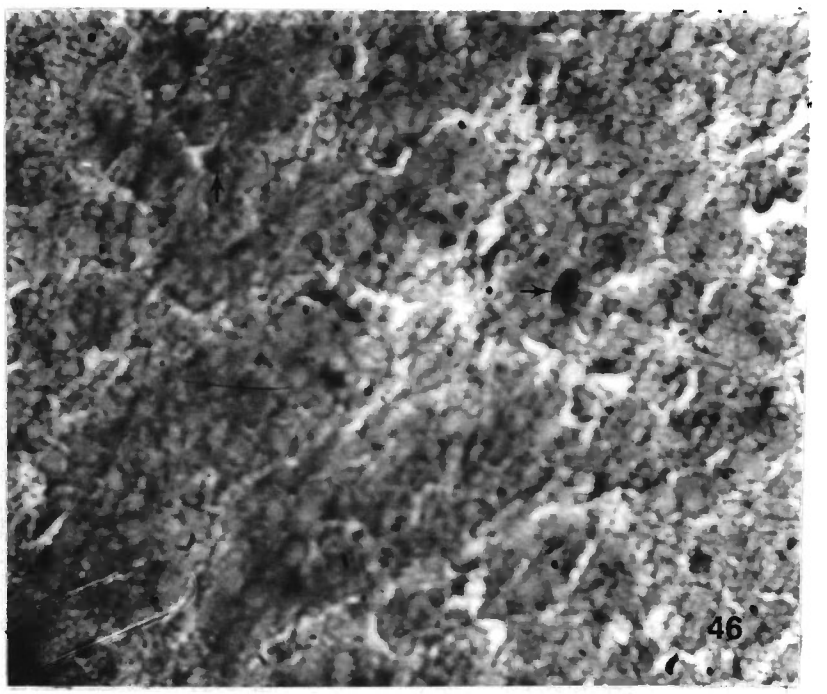
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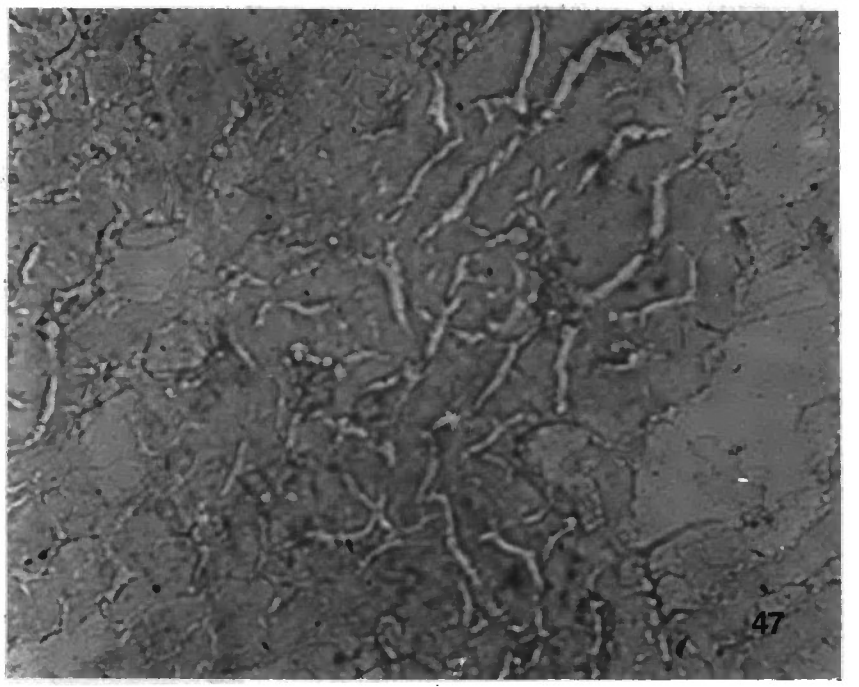
Fig. 47. Photograph of control slide showing negative reaction for cytochrome oxidase. 45X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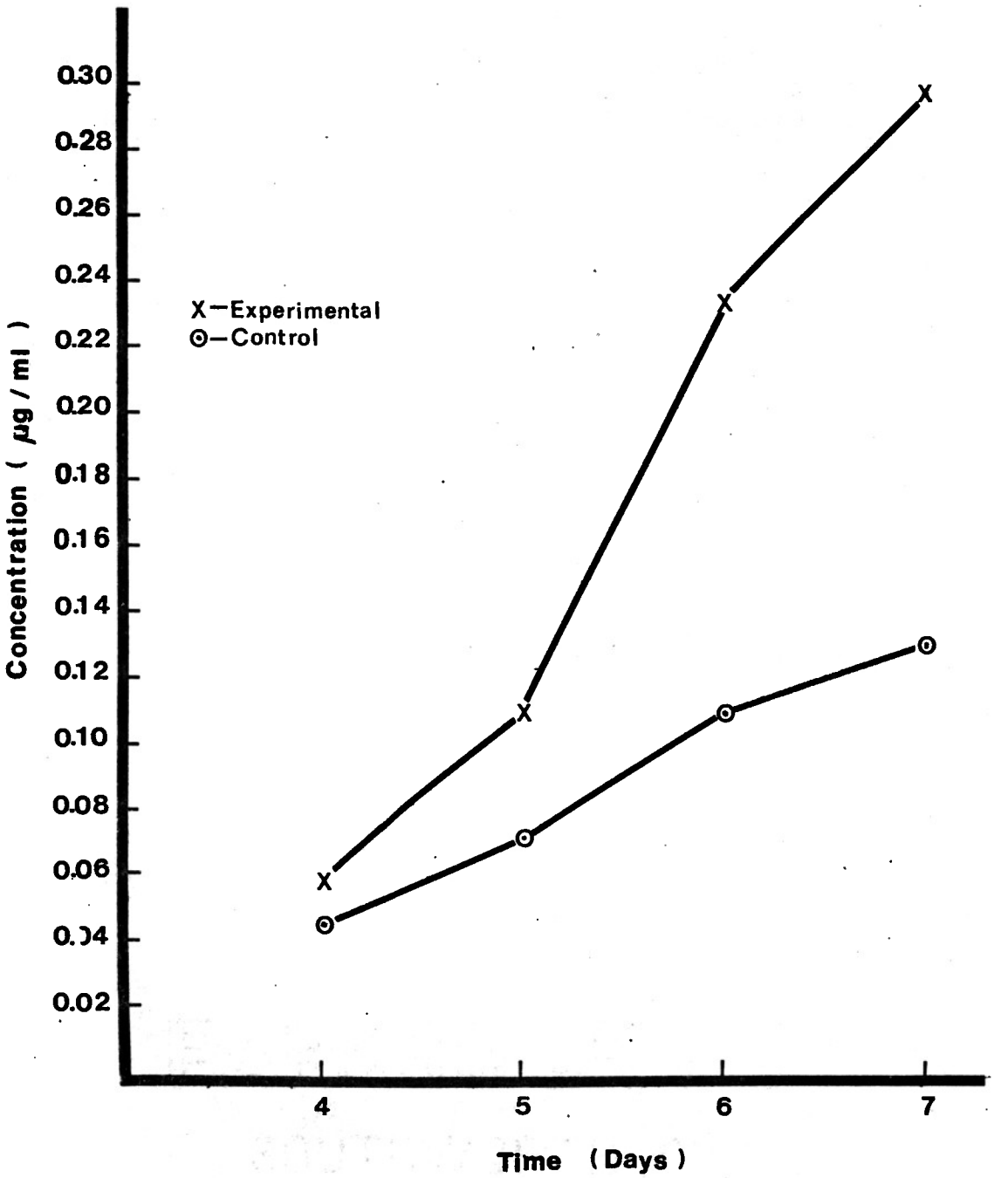
Atomic Absorption Determinations

Values for known concentrations of cadmium standards were plotted against absorbancy readings. All absorbancy readings for experimental and control organs fell in the lower half of the graph for the standard curve. Hence, this portion of the standard curve was magnified to determine the concentrations in µg/ml of experimental and control organs.

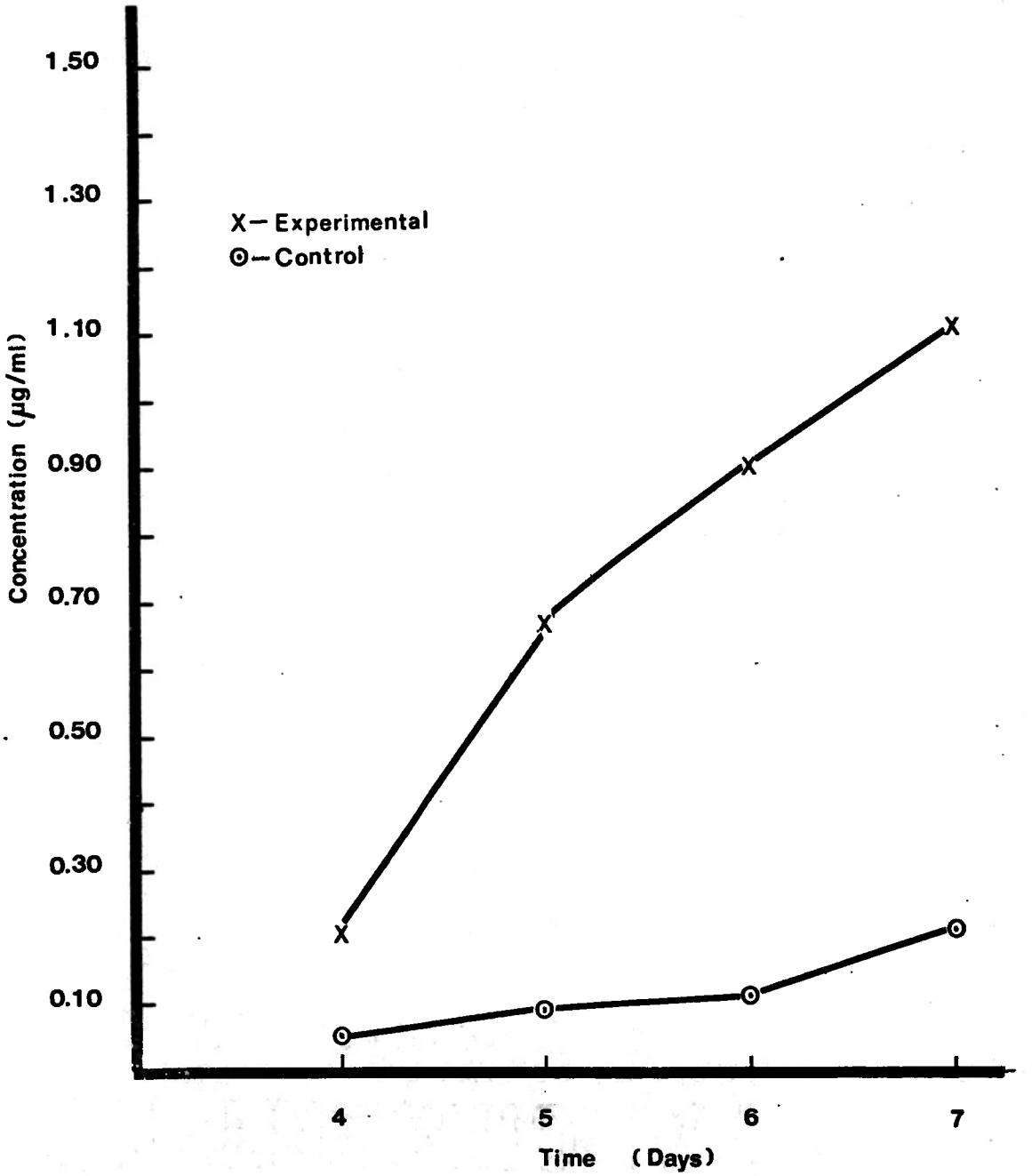
The concentrations of cadmium in the liver and kidneys of cadmium-treated embryos are shown graphically in Figs. 48-49. In Fig. 48 the data from investigations of cadmium concentrations in liver (µg/ml wet weight) in relations to age (days post-incubation) have been assembled for control and cadmium-treated embryos. As can be seen, the values for controls are comparatively low and do not exceed 0.130 µg/ml. The values for cadmium-treated liver are considerably higher showing values of 0.297 µg/ml in 7-day embryos. As the cadmium concentrations in the liver of 7-day embryos are greater, it is obvious that there is an accumulation with age.

In Fig. 49 values for mesonephric tissue are plotted in relations to time (age). From this graph one sees that the control values are lowest, but even these show an accumulation with time (4-day, 0.059 µg/ml; 7-day, 0.211 µg/ml). Kidneys from cadmium-treated embryos are extremely higher than controls (6-day, 0.904 µg/ml). Experimental kidneys on an average are about 50% greater in concentration than control kidneys. Concentrations also increase with age in this organ, with values ranging from 0.204 µg/ml for 4-day to 1.65 µg/ml for 7-day embryos.

Fig. 48. Graph showing concentration of cadmium in liver of cadmium-treated embryos.


0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
Concentration (µg / ml)
X—Experimental
⊙—Control
4
5
6
7
Time (Days)

Fig, 49. Graph showing concentration of cadmium in kidneys of cadmium-treated embryos.


1.50
1.30
1.10
0.90
0.70
0.50
0.30
0.10
Concentration (µg/ml)
X— Experimental
⊙— Control
4
5
6
7
Time (Days)

In Fig. 50 the cadmium concentrations of experimental liver and kidneys are graphically compared as % increase. From this graph two important points can be determined: (1) the % increase of experimental organs (liver and kidneys) over control organs, and (2) the comparative difference % increase between the liver and kidneys. In the case of liver tissue, 4-day embryos showed a 28% increase and 7-day embryos showed a 128% increase over the values for controls. Kidney tissue showed % increases as high as 681% (7-day embryos). Therefore, the average % increase was considerably higher than that of controls. All values for per cent increase of liver and kidneys are shown in Table 3.

Fig. 50. Graph comparing cadmium concentrations of experimental liver and kidneys with values expressed in per cent increase.

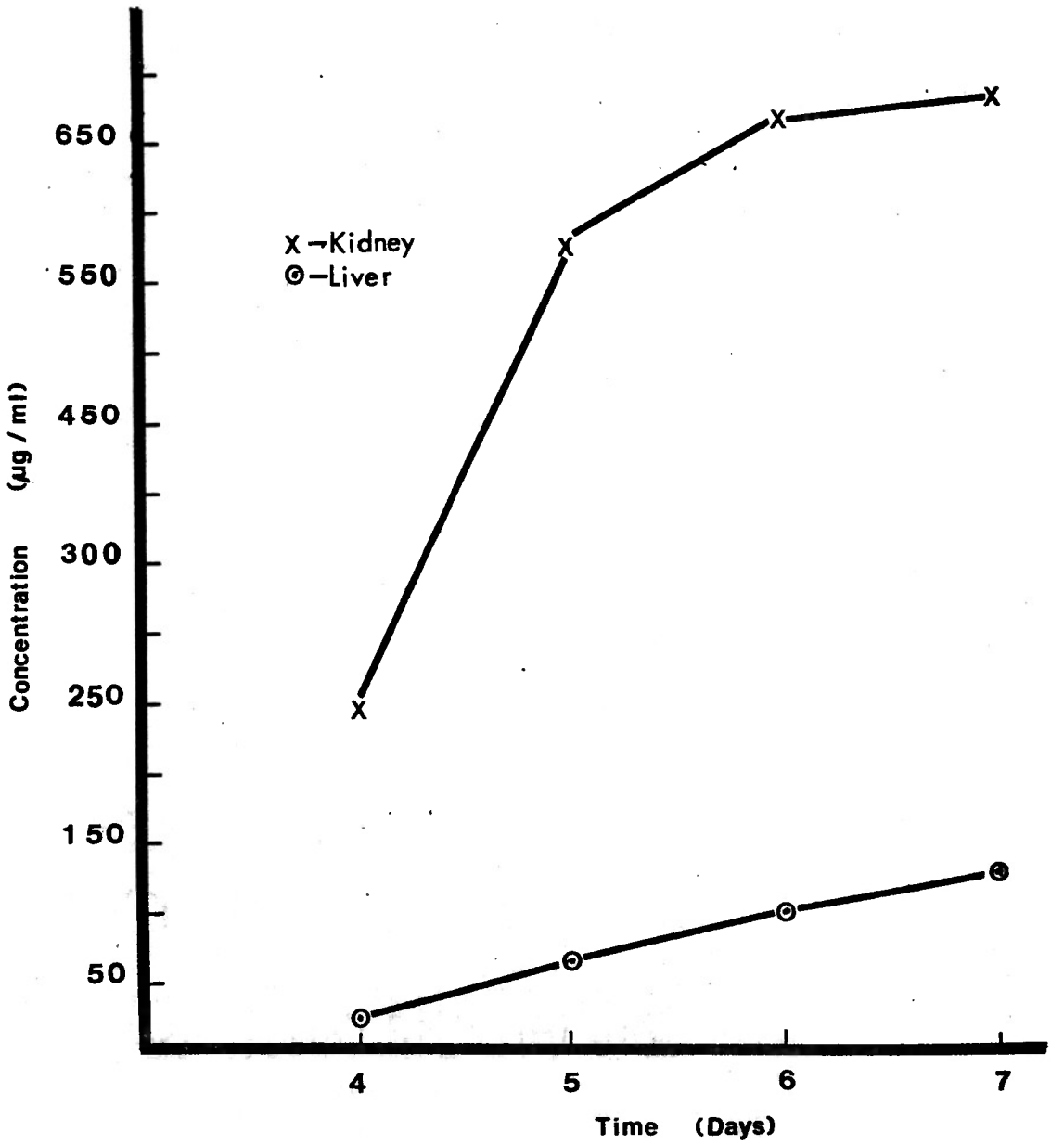

650
550
450
300
250
150
50
Concentration (µg / ml)
X—Kidney
⊙—Liver
4
5
6
7
Time (Days)

Table 3. Concentration of cadmium in the liver and kidneys of chicken embryos[a]

Organ	Age of Embryos			
	4-day	5-day	6-day	7-day
Liver	28%	63%	100%	128%
Kidneys	245%	572%	666%	681%

[a]Values expressed in per cent increase.

CHAPTER V

DISCUSSION

The results presented in this investgation have shown that administration of cadmium to chicken embryos may result in pronounced damage to the renal tubular structure. Slight swelling of epithelial cells and ruptured blood vessels of the mesonephros were recognized in earlier stages with dage continuing to increase with post-incubation time. These changes were consistent with those reported in the rat (Bonnell et al., 1960), rabbits that suffered renal damage in Cd poisoning (Ahlmark et al., 1961), and those which have been found in poisoning with other metals in addition to cadmium (Mottet, 1974). The deposits of cadmium were accompanied by toxic alterations in the tubular epithelium. The extent of these lesions appeared to be directly related to the amount of cadmium deposited.

The histological examination of mesonephric changes in the present study also revealed degeneration of the glomeruli. The distinct space that is normally between the glomeruli and Bowman's capsule became filled with dispersed cells as a result of damage to the glomeruli. In contrast, Axelsson et al., (1968) reported that renal lesions in cadmium-exposed rabbits were localized only in tubules and that the glomeruli were normal. The findings presented here, however, are in accordance with Bonnell et al., (1960) who reported that renal lesions in rats consisted of distortion of tubules and secondary ischemic changes in the glomeruli. Renal lesions, were also observed by Itokawa et al., (1974), in both

tubules and glomeruli. Thus, the finding of this investigation may be correlated with the functional disorder of the kidneys found by others (Friberg, 1950; Kazantzis et al., 1963). That is, these functional disorders of the kidneys of Cd-poisoned organisms may originate from lesions in the proximal tubules, and glomerular lesions are possibly secondary lesions resulting from tubular dysfunction.

When comparing Cd-treated embryos to control organisms of the same age, there is a distinct difference in the degree of renal damage between the two, with the greatest being in the Cd-treated ones. These findings are not in agreement with those of Saxon and Kimball (1941) who describes tubular damages as ageing changes in rats rather than the result of cadmium toxicity. The reason for these conflicting interpretations may lie in the fact that although ageing is taking place in both cases, this work deals with an embryonic system while the other investigations are using adult systems.

Site-specific teratogenic stimuli have been of increasing interest in experimental embryology. The fact that certain teratogens will affect the development of specific organs or tissues in embryos has been well demonstrated (Fraser et al., 1954). The ontogeny of a particular malformation, however, must be related to the site specificity of the timing of the insult involved as well as the timing of that insult during organogenesis. One interesting concept concerning this point is that in the course of normal organogenesis there are definite periods at which the threat of a teratogenic stimulus would end. Thus, for example, in the

true sense of the term teratogenic, it would not be possible for limb abnormalities to occur after administration of a teratogenic stimulus once the limb has properly formed. At the other end of the time spectrum little evidence exists for the concept that a teratogen may cause malformations during very early stages of development. Some teratogens may be present within the maternal system during critical stages of development (Ingalls, 1954). This has led us to suggest that the time-related effect that Cd had upon the chick embryonic kidney was in accordance with the functional development of this organ. According to Romanoff (1960) the embryonic mesonephros does not start functioning until around day 5 or 6. The results of this study showed a greater degree of renal damage at 6-7 days of post-incubation than earlier although the mesonephros does begin functioning before this stage.

The preferential occurrence of Cd in the mesonephros of chicken embryos in this investigation may be accounted for by the Cd-binding protein, metallothionein. Metallothionein was first isolated from equine renal cortex by Margoshes and Valle (1957). Further purification showed it to contain 5.9% Cd and 2.2% Zn. The demonstration of this protein in the human renal cortex (Pulido et al., 1966) and its similarity to that of the horse lends support to the hypothesis that analogous proteins will be found in yet other species.

In previous investigations (Harris and Hunter, 1977) concerning the effects of Cd and Zn on chick embryonic development, a significant Zn-Cd interaction was noted. The mortality was considerably less in those receiving zinc and greater in those receiving cadmium. However, the degree

of hemorrhaging and rupture was decreased by simultaneous injections of Zn and Cd, and was presumably due to the antagonism of cadmium and zinc. These findings lend support to the work of Schroeder (1964) who found that cadmium accumulates in the kidney and liver through life, and since an elevated ratio of Cd to Zn commonly occurs in hypertension, this has been suggested as a factor in pathogenesis of the disease. Physiological and biochemical studies have indicated that Cd administration alters normal renal tubular function and decreases sodiun excretion and glucose reabsorption (Lener and Musil, 1970).

Morphological findings of Kanisawa and Schroeder (1969) indicate that chronic Cd administration produces constriction of smaller renal arteries, mild dilation of larger arteries and diffuse fibrosis of capillaries at even low dose levels. These changes would suggest a decrease in effective renal circulation with time, which may support the increase in renal damage with time seen in the present investigation.

Results of this study also showed the greater portion of the tubular epithelium disarranged and a mass of red blood cells in the mesonephros of selenium-treated embryos. However, comparative examination revealed that destruction in selenium-treated embryos was not as great as that of cadmium. Muth and Binns (1964) have shown that compounds containing selenium at different levels in the diet can either be hepatotoxic and hepatocarcinogenic or act as essential nutrients for several mammalian and avian species. Inorganic selenium compounds occur naturally in many soils and the element is incorporated into plant protein, partly as

selenomethionine and selenocystine. In some areas the forage contains high amounts of organic selenium compounds and is toxic to livestock. In other areas the soil is deficient in selenium compounds and livestock fed on forage grown in these areas develop selenium-deficiency disease. In several mammalian and avian species, selenium compounds act as essential dietary constituents (Oldfield et al., 1963).

On the other hand, in the rat, selenium in the form of selenide, selenate, or seleniferous corn or wheat, appears to be hepatocarcinogenic when fed in the diet for many months (Nelson et al., 1943). This is possibly an implication for the present investigation in that the dosage or form of selenium administered may not have been tolerable at this embryonic level.

Selenium prevents lethality and testicular injury from Cd even though it causes a marked increase in Cd levels in blood and testis (Gunn and Gould, 1967). However, the fact that Cd augments levels of selenium in the blood and testis and also disminishes its excretion (Ganther, 1962), suggests some sort of binding between the two elements in which Cd is rendered innocuous. Considering the ratio of protective agent to Cd which is needed, previous investigations (Harris and Hunter, 1978) and the present report led us to suggest that selenium and zinc are relatively more efficient in protecting against letality than preventing injury from cadmium.

Unlike some organs that reach full functional maturity during fetal life and remain inactive until shortly after birth, the chicken embryo

mesonephros will later bereplaced by the definitive metanephros-which will persist throughout postnatal life. An indirect correlation between morphological development and physiological function is implied from the preceding experiments, but morphological evidence is not adequate enough to evaluate the chemistry of the organ. The application of histochemical methods to the problem of functional development yields evidence concerning the chemical nature of the processes involved. The major premise inchemical embryology is that morphogenetic chenges are basically chemical changes and are therefore amendable to some type of chemical analysis and interpretation (Burstone, 1962). Therefore, by applying histochemical methods to kidney sections from chick embryos we were able to gather additional information as to the effect of heavy metals on embryonic development.

The present investigation shows that cadmium chloride affects the embryonic chick in such a way that the normal developmental occurrence of acid and alkaline phosphatase is altered. The results presented give descriptive evidence that Cd elevates the quantity of acid phoaphatase and inhibits alkaline phosphatase activity in developing chick embryos from 4-7 days post-incubation. These results imply that Cd disturbs the quantity of acid and alkaline phosphatase in chick embryos as presented by Moog (1944). She found that both acid and alkaline phosphatase are present in the unincubated blastoderm of the hen's egg, and all embryonic tissues during the first two to four days of development, the

concentration of alkaline phosphatase being much greater than that of acid phosphatase.

The literature is well documented as to the presence and localization of alkaline phosphatase. Desalu (1965), using an azo-dye technique, localized alkaline phosphatase in the metanephric kidneys of fetal rats along the luminal (brush) border of the differentiated proximal tubule. These findings correlate with the evidence in the present investigation as far as alkaline phosphatase localization is concerned. The brush borders of proximal tubules in the control mesonephros demonstrated a much more intense reaction of alkaline phosphatase activity than in any stage of experimental (Cd-exposed) embryos. Results of this investigation also revealed inhibition of cytochrome oxidase activity in the membranes of tubular structures. In comparison, this reduction in Cd-exposed embryos is supported by Axelsson and Piscator (1966) who found a reduction in alkaline phosphatase activity after exposing rabbits to Cd for five days a week. Hill et al., (1963) reported that, while studying the interactions of Cd and Zn, whenever Cd was fed alone, the cytochrome oxidase of heart homogenates was depressed. Many investigators have shown correlations between alkaline phosphatase activity and membrane transport (Danielli, 1953) and between cytochrome oxidase and oxidation activity (Lehninger, 1965). This led to the implication that inhibition of alkaline phosphatase and cytochrome oxidase as was seen in Cd-treated embryos of this investigation, correlated with alteration of membrane transport and decrease oxygen consumption due to cell death.

The present investigation also showed that tubular epithelium gave positive reactions for beta-glucuronidase, with activity becoming

progressively stronger toward the lumen of the tubule. Nishizumi (1972), in an electron microscope study of cadmium nephrotoxicity in rats, found an increase in the number of lysosomes and autophagic vacuoles. This phenomenon became more prominent with time in the respective experimental group, and with an increase in concentrations of cadmium in the same experimental period. Exact functions of lysosomes are not fully elucidated, but these organelles are known to be concerned with the segregation and, whenever possible, the degradation of substances taken up by cells from the environment as well as cytoplasmic constituents (de Duve and Wattiaux, 1966). The significance of this increase may be evaluated in relations to the increase that we noted in acid phosphatase and beta-glucuronidase, since hydrolytic enzymes of most tissues have been shown to occur inside lysosomes (de Duve, 1957; Baudhin et al., 1964; Beaufay et al., 1964).

Atomic absorption spectrophotometry proved particularly useful for the work with cadmium content and its concentration in embryonic kidneys and liver. The mesonephros of cadmium-exposed embryos contained from three to four times as much cadmium as the liver. These findings are in accordance with those of Stitch (1957) who estimated spectrographically the cadmium content of tissues obtained at necropsies of people who died as the result of accidents and had not been occupationally or experimentally exposed to cadmium. The kidneys of these individuals were found to contain the highest concentration of Cd, suggesting that they selectively retained cadmium. The results of the present investigation

are also supported by Chaube, Nishimura, and Swinyard (1973) who by analysis of liver and kidneys using atomic absorption showed Cd concentrations in the kidneys to be approximately three times greater than that of liver.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Injections of cadmium chloride ($CdCl_2$), 0.25 cc of a 0.025 M, were given to an experimental series of 48 hr chick embryos.
2. The gross visible effects which followed were hemorrhage along the periphery of the cranial lobes and optic cups as well as along the neck.
3. Microscopical studies on cadmium-treated embryos showed that the degree of renal damage was not as extensive in the 4- and 5-day chick embryos as that in the 6- and 7-day ones. There was a shedding of cell nuclei towards the tubular lumen and in some instances the greater part of the tubular structure was effaced, with only the tubular wall remaining. Marked degeneration was also observed in the glomeruli.
4. Injections of sodium selenite into chick embryos were made using the same dosage and concentration at the time cited above. The tubular epithelium was disarranged and a mass of red blood cells was detected in the mesonephros but destruction in the majority of selenium-treated embryos was not as great as that of cadmium.
5. Associated with the rupturing of the visceral lining were hematomas and a profusion of red blood cells where the wall separated from the visceral organs. Necrotic lesions were irregularly distributed throughout the liver. Blood cysts,

areas of destruction of the parenchyma, filled with recently extravasted erythrocytes, were commonly observed.

6. In five-day post-incubation kidneys, the overall amount of acid phosphatase activity in the lesions was much reduced by comparison with the seven-day-old specimens. At six-day post-incubation, the acid phosphatase activity had attained a widely dispersed distribution, in which areas of intense activity were isolated as islands surrounded by regions in which the reaction was either weak or negative. At seven-day post-incubation there was no delineation of cells in the tubular epithelium.
7. At six-days post-incubation in cadmium-treated embryos, finely granular evenly dispersed deposits of alkaline phosphatase activity were present in both the proximal and distal areas. The brush-borders of proximal tubules in the control kidneys demonstrated a much more intense reaction of alkaline phosphatase than in any stage of experimental embryos. The activity was in the majority of cases so intense that the lumen of the tubules was almost completely obscured.
8. Tubular epithelium gave a slightly positive reaction for beta-glucuronidase activity, becoming progressively stronger toward the lumen of the tubule. Kidney tubules at sites of damaged tissue gave an intense reaction in

some cases, with material filling the lumen of the tubules. The finely granular reaction differed from acid phosphatase reactions in that a more discrete type was seen in the tubules.

9. As in the case of alkaline phosphatase, the cytochrome oxidase activity was more observable in the control section than in the experimental. Sections from six-day control embryos showed frequent enzyme positive granules in the membrane of tubular structures. Liver tissue of control embryos showed a more intense staining of hepatic cells when compared to mesonephric tissue.
10. Histochemically, alkaline phosphatase and cytochrome oxidase are inhibited by cadmium chloride, while acid phosphatase and beat-glucuronidase are elevated in the presence of cadmium.
11. The concentrations of cadmium in the liver and kidneys of cadmium-treated embryos were analyzed by atomic absorption spectrophotometry. The mesonephros of cadmium-treated embryos contained from three to four times as much cadmium as the liver.

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